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DOES GLIADIN CONTAIN AMIDE NITROGEN?*

By THOMAS B. OSBORNE and OWEN L. NOLAN.

(*From the Laboratory of the Connecticut Agricultural Experiment Station, New Haven.*)

(Received for publication, July 1, 1920.)

The Origin of Ammonia Nitrogen in Gliadin.

The amount of ammonia which various proteins yield when hydrolyzed with acids was shown some years ago in a paper[1] from this laboratory to correspond closely to that required for amide combination with the sum of the glutaminic and aspartic acids which these also yield. That the combination $R\text{-}CONH_2$ actually occurs in the protein molecule is not proved by the agreement between the values found by analysis and those calculated, but this agreement was so close that the suggestion then advanced has been generally accepted as probably correct. Recently Thierfelder and von Cramm[2] have confirmed the evidence just referred to, and contributed further support for this view by experiments with synthetic peptides containing glutamine, as well as with glutamine itself. Additional evidence that the nitrogen which is converted into ammonia by boiling proteins with acids is actually in amide combination is furnished by new experiments which we have just completed.

These were made on the assumption that as $R\text{-}CONH_2$ groups yield free carboxyl groups on hydrolysis the residual products should reveal their presence by an equivalent increase in acidity. Preliminary experiments showed that boiling gliadin with 1 per cent hydrochloric acid for 2 hours yields almost as much ammonia

*The expenses of this investigation were shared by the Connecticut Agricultural Station, and the Carnegie Institution of Washington, D. C.

[1] Osborne, T. B., Leavenworth, C. S., and Brautlecht, C. A., *Am. J. Physiol.*, 1908–09, xxiii, 180.

[2] Thierfelder, H., and von Cramm, E., *Z. physiol. Chem.*, 1919, cv, 58.

as boiling with 20 per cent acid for 24 hours. The slight increase in free amino nitrogen which occurs during this mild hydrolysis shows that a relatively small proportion of the peptide bindings are broken. Since upwards of 90 per cent of the maximum obtainable ammonia is yielded at a rate strictly proportional to the time of boiling as well as to the concentration of the acid it seems fair to assume that nearly all the ammonia originates from a common group which, like $R\text{-}CONH_2$, is very easily hydrolyzed.

Accordingly 5 gm. of air-dry gliadin, equal to 4.7 gm. dried at 110° and containing 0.8125 gm. of nitrogen, were boiled with 500 cc. of 1 per cent hydrochloric acid for 1 hour. The gliadin at first dissolved in the boiling acid, but soon began to separate, finally forming a coherent mass. At the end of the hour 244 cc. of a solution containing 4.9776 gm. of sodium hydroxide were added. The strongly acid solution was vigorously shaken for some time and the suspended solids were then removed by centrifuging and the solution was made up to 1,000 cc. Analysis of aliquot parts showed that this solution contained 9.1720 gm. of solids, of which, after ignition at a low red heat, 7.3770 gm. remained as ash. It also contained 0.1568 gm. of nitrogen as ammonia, equal to 0.8381 gm. of NH_4Cl, if all the ammonia was present as this salt. The total inorganic matter was, therefore, equal to 8.2151 gm., leaving 0.9569 gm. of organic solids in the solution, or 20.3 per cent of the gliadin. The solution contained 0.3171 gm. of N, of which 0.1568 gm. was ammonia nitrogen, the non-ammonia nitrogen consequently being 0.1603 gm., equal to 16.8 per cent of the organic solids. Since in this experiment only 78.0 per cent of the maximum amount of the ammonia obtainable by complete hydrolysis was liberated it is probable that some unchanged gliadin remained dissolved in the acid aqueous solution. This would account for the higher nitrogen content of the soluble organic solids than that calculated for gliadin from which all its amide nitrogen had been split off.

To neutralize 100 cc. of this solution required 0.01428 gm. of NaOH, equal to 0.1428 gm. for the entire solution. The solids removed by centrifuging were suspended in water, and 0.2683 gm. of NaOH was added gradually with vigorous shaking. This was a little more than enough to dissolve all the solids and just sufficed to make the solution neutral to litmus. When 0.0377

gm. more of NaOH was added the reaction became just alkaline in phenolphthalein. In neutralizing the solution and precipitate there were thus added 4.9776 + 0.1428 + 0.2683 + 0.0377 = 5.4264 gm. of NaOH equivalent to 4.9448 gm. of HCl. Since the ammonia formed by hydrolysis also neutralized a part of the HCl with which the gliadin had been boiled the total base available for neutralizing acid was equivalent to 0.4551 gm. more NaOH, thus making the total equal to 5.8815 gm. Subtracting from this the NaOH required to neutralize the 5 gm. of HCl, namely 5.4869 gm., leaves 0.3946 gm. of NaOH which had been neutralized by acid groups formed during hydrolysis. Since the amount of ammonia found in the solution after hydrolysis was equivalent to carboxyl requiring 0.4551 gm. of NaOH for complete neutralization the amount of NaOH which neutralized the products of hydrolysis to phenolphthalein was 86.7 per cent of the amount calculated for a complete reaction with all the R-COOH groups which would have been formed if all the ammonia originated from R-$CONH_2$ groups. The acidity developed therefore corresponds to carboxyl groups nearly, if not quite, equivalent to the ammonia produced by hydrolysis.

Since the sum of the amounts of glutaminic acid and aspartic acid which has been obtained from gliadin, the amount of ammonia liberated by hydrolysis, and the increased acidity accompanying the formation of ammonia all correspond closely with the quantities required for nitrogen in amide union with one carboxyl of each molecule of the di-basic acids, there can be little doubt that the ammonia nitrogen is present in the gliadin molecule in amide union with one of the carboxyl groups of each of the glutaminic and aspartic acid groups.

Recently a new question has been raised in connection with this problem by Dakin's[3] discovery that some proteins contain oxyglutaminic acid in relatively considerable quantity. This acid might, therefore, afford additional carboxyl groups for a possible amide union. In this connection it is of interest to note that Andersen and Roed-Müller[4] have described a method whereby the carboxyl groups belonging to di-basic monoamino-acids can be determined in the mixed products of hydrolysis of

[3] Dakin, H. D., *Biochem. J.*, 1918, xii, 291.

[4] Andersen, A. C., and Roed-Müller, R., *Biochem. Z.*, 1916, lxxiii, 326.

proteins. The ammonia and basic amino-acids are removed from the products of hydrolysis and amino nitrogen is determined in an aliquot part of the solution. Another part of the solution is neutralized by Sörensen's formol method, evaporated, and ignited. By determining the carbonate in the ash and calculating its ratio to the amino nitrogen the excess above a 1:1 ratio measures the proportion of dicarboxylic acids. By applying this method to gliadin they conclude that 24.33 per cent of its nitrogen belongs to such acids, whereas the glutaminic and aspartic acids which Osborne and Guest[5] obtained contained 24.0 per cent. The average of numerous accordant determinations of ammonia nitrogen, namely 24.4 per cent of the total gliadin nitrogen, is in close agreement with the above figures. If gliadin yields appreciable quantities of oxyglutaminic acid this should have been detected by Andersen and Roed-Müller's method. These investigators, on the other hand, found that casein contains an amount of monoaminodicarboxylic acid equivalent to 17.7 per cent of the total nitrogen. This is very much more than Osborne and Guest[6] found in casein, for the sum of the glutaminic and aspartic acids which they obtained contained nitrogen equivalent to only 10.4 per cent of the casein nitrogen. Foreman,[7] later, obtained 21.8 per cent of glutaminic acid from casein, a figure since confirmed by Dakin who found in addition 4.1 per cent of aspartic acid and also 10.5 per cent of oxyglutaminic acid. Dakin's results show that casein yields di-basic monoamino-acids containing nitrogen equal to 22.3 per cent of its total nitrogen, or more than twice as much as corresponds to the maximum amount of ammonia yielded by complete hydrolysis. Even omitting Dakin's oxyglutaminic acid, these newer determinations of glutaminic and aspartic acid show the presence of an excess of di-basic acids 50 per cent greater than would suffice for amide combination with the ammonia nitrogen. The marked acidity which, in contrast with most other native proteins, is characteristic of casein may possibly be thus explained.

Andersen and Roed-Müller point out that if all the monoamino-acids in the protein molecule are in peptide union and the di-basic

[5] Osborne, T. B., and Guest, H. H., *J. Biol. Chem.*, 1911, ix, 425.
[6] Osborne, T. B., and Guest, H. H., *J. Biol. Chem.*, 1911, ix, 352.
[7] Foreman, F. W., *Biochem. J.*, 1914, viii, 463.

monoamino-acids occur as amides the products of hydrolysis should be basic, since the carboxyl and amino groups should balance one another, leaving the basic groups of the diamino-acids unneutralized. They say:

"In order to obtain neutral reacting products one must assume either that some unions occur in which more basic than acid groups have disappeared, or that nitrogen-free acids occur together with the amino-acids. It is not clear how, in the first case, the bindings can be represented. If unions of this kind occur a neutral body on hydrolysis must yield a basic reacting mixture of amino-acids which, after digestion with trypsin and erepsin, must be noticeable. . . . . We, therefore, do not believe in the existence of such bindings in the protein molecule. . . . . The second assumption, that nitrogen-free acids are present also is not probable, because, in the many investigations known to us which have been made of the composition of the proteins, such acids have never been isolated, with the exception of carbonic acid and very small amounts of methyl sulfuric acid."

Andersen and Roed-Müller then show that no volatile nitrogen-free acid except carbonic is yielded by casein. Believing that these considerations raise a question as to how the ammonia nitrogen is actually bound in the protein molecule, they say: "The assumption of the presence of all the ammonia as equivalent to the monoaminodicarboxylic acids and united with these, is not in harmony with the properties of the proteins." This conclusion of Andersen and Roed-Müller does not have the force that they attribute to it because the products of hydrolysis of proteins form a buffer solution in which an excess of alkali, or acid, would have but little effect in changing the reaction. Andersen and Roed-Müller[8] believe that the experiments of Skraup and von Hardt-Stremayr,[9] and also those of Denis[10] indicate that all the ammonia nitrogen cannot be present in amide combination. These investigators state that ammonia is not liberated at a uniform rate when proteins are boiled with strong acid, but that the last part is set free gradually and that the maximum amount is not produced until after the biuret reaction has vanished and the hydrolysis has been completed, or nearly completed.

[8] Andersen, A. C., and Roed-Müller, R., *Biochem. Z.*, 1915, lxx, 442.

[9] Skraup, Z. H., and von Hardt-Stremayr, E., *Sitzungsber. k. Akad. Wissensch. Math-naturw. cl. Wien., 2te Abt*, 1908, cxvii, 25.

[10] Denis, W., *J. Biol. Chem.*, 1910, viii, 432.

Glutamine and asparagine on the other hand yield all their amide nitrogen after a brief treatment with hot dilute acid. They, therefore, consider the possibility of uramino-acids as a source of a part of the ammonia nitrogen.

Experience has convinced us that, at least, in the case of gliadin, or of edestin, all but an insignificant part of the ammonia nitrogen is liberated by boiling for a comparatively short time with very dilute acid, while at the same time only a small part of the peptide bindings are set free. We are also convinced that the slightly higher figures for ammonia, which others have obtained by subjecting proteins to severe treatment with acids, compared with the lower figures obtained after a milder treatment are to be ascribed to secondary decomposition of some of the amino-acids.

Thus we have found that boiling gliadin with 1 per cent HCl for 2 hours converted 23.6 per cent of the gliadin nitrogen into ammonia and only 3.7 per cent into free amino nitrogen, while after 5 hours these figures were 24.2 and 9.0 per cent, respectively. The average maximum yield of ammonia after boiling gliadin with 20 per cent HCl for 24 hours is very little greater; namely, 24.4 per cent of the total nitrogen. These figures show that 97 per cent of the ammonia nitrogen is liberated before more than a small proportion of the peptide bindings has been broken.

Uramino-acids are converted into hydantoins by boiling with dilute acids and yield ammonia only when boiled with strong acids for a long time. The uramino group,[8,11] as a source of more than a very small part of the ammonia nitrogen of gliadin, is thus excluded.

SUMMARY.

When gliadin is boiled with dilute acid a degree of acidity is developed which is closely proportional to the amount of ammonia, thus indicating that ammonia originates from R-CONH$_2$ groups.

Since very nearly as much ammonia is produced by boiling for a short time with 1 per cent HCl as by boiling for 24 hours with 20 per cent HCl the presence of more than extremely small amounts of uramino groups, R-CH-NH-CO-NH$_2$, is excluded.

[11] *Cf.* also Lippich, F., *Z. physiol. Chem.*, 1914, xc, 441.

METHODS USED IN DETERMINING THE ELECTRIC CONDUCTIVITY OF SOLUTIONS.*

By J. F. McCLENDON.

(From the Physiological Laboratory of the University of Minnesota Medical School, Minneapolis.)

(Received for publication, June 11, 1920.)

In making a study of the permeability of bacterial membranes it was thought advisable to look into the principles of the methods used in determining electric conductivity of solutions, since they applied to the study of suspensions of bacteria. The method generally used requires an alternating current, Wheatstone bridge, and telephone for detecting the current. The solution is placed in a conductivity cell containing two metallic plates or electrodes. Since the electrodes are usually made of platinum, and platinum salts are absent from the solution, polarization occurs at the electrodes and no considerable current can pass in one direction without a potential difference across the phase boundary equal to the decomposition potential of the water or salts composing the solution. Since the decomposition potential of water is 1.7 volts and potentials of various salts fluctuate around this figure, a counter E. M. F. of about 1 or 2 volts is added to the passive resistance of the solution and prevents the estimation of the latter. In order to obtain correct results, no current should be allowed to pass between the electrodes and the solution. The electrode surface and solution surface constitute an electric condenser of enormous capacity, since the capacity of a condenser varies inversely with the distance between the plates and this distance is very small, being the thickness of the electric double layer at the metal-water surface. By increasing the frequency of the alternating current and increasing the surface area of the electrodes a point may be reached at which the impedance caused

* Partial report on work done under a grant from the United States Interdepartmental Social Hygiene Board.

by the phase boundary is so small as not to interfere with results of considerable accuracy. Alternating currents of high frequency may be produced by means of Vreeland oscillators, and by vacuum tube oscillators as used in wireless transmission. The electrode surface may be increased by enlarging the electrode or roughening it. An extremely rough electrode surface may be obtained by rapid electroplating in a solution of platinic chloride containing a trace of basic lead acetate. This platinized surface is so rough that it does not reflect light into the eye and hence appears black. Platinized electrodes have an enormous capacity when freshly prepared, but the capacity may decrease with use. This decrease is probably due to the interposition of a poorly conducting layer between the metal and the solution due to adsorption of colloids, or to stoppage of the pores in the surface, thus decreasing the area acting as a condenser. The capacity of a dirty electrode may sometimes be restored by cleaning it with a saturated solution of a bichromate in concentrated sulfuric acid. If this fails, the electrode must be platinized again.

In some of our experiments the electrodes decreased in capacity while the bacterial suspension remained in the conductivity vessel, and cleaning or platinizing of the electrode would necessitate complete change in experimental procedure. In such cases, not only is the impedance greater than the resistance that it is desired to measure, but a tone silence in the telephone cannot be obtained owing to the fact that the current is not in phase with the voltage across the conductivity cell. In recent years, a compensation method for obtaining a tone silence or sharp tone minimum in the telephone has been widely advocated in this country. The method usually consists in the placing of a variable condenser in parallel with the resistance box in the ordinary Wheatstone bridge apparatus (in which the two other resistances are represented on a meter wire with sliding contact). This method enables the operator to obtain a sharp tone-minimum and express his results in figures with five decimal places. It is one of the objects of this communication, however, to emphasize that such figures may be incorrect in the second place.

Our experiments have been made with frequencies varying continuously from 60 to 500,000 cycles per second produced by a Vreeland oscillator and a vacuum tube oscillator made of a West-

ern Electric, VT 2, bulb. At radio-frequencies, we have had to simplify the apparatus in order to obtain currents in phase with the voltage and have had to eliminate much of the Wheatstone bridge apparatus. Furthermore, we used a crystal detector and galvanometer and have never achieved the accuracy possible with the lower frequencies and telephone. This paper will be confined, therefore, to work with the Vreeland oscillator and telephone receiver.

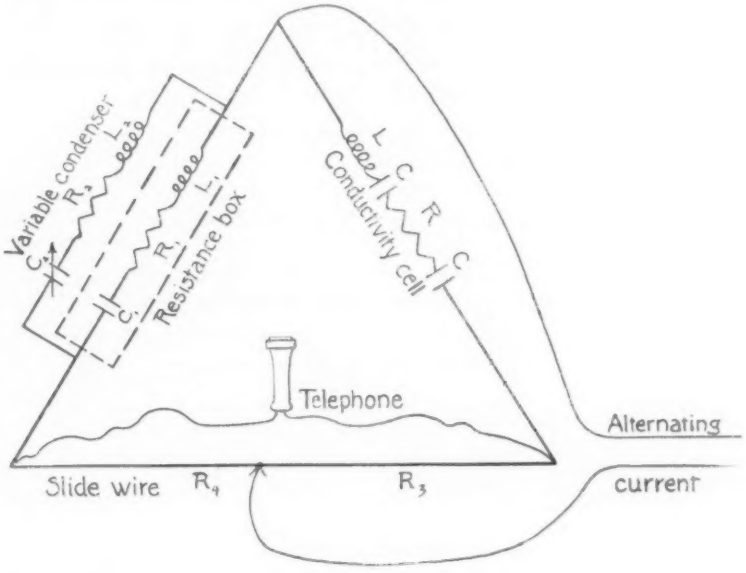

FIG. 1.

Our Wheatstone bridge is diagramed in Fig. 1, in which resistance is denoted by R, capacity by C, and inductance by L. In the conductivity cell, the resistance, R, is the solution; the total capacity is one-half the capacity of one electrode surface, C; and the inductance, L, is in the connecting wires. By straightening these wires, the inductance may be reduced to a small figure. The resistance box has a variable resistance, R_1, a small capacity in parallel but an infinite capacity in series, C_1, and a small inductance, L_1. In parallel with the resistance box is a variable

condenser with variable capacity, C_2, small resistance, R_2, and small inductance, L_2. In the cell arm of the bridge, given the resistance in ohms, the capacity in farads, and the inductance in henries, the impedance, Z, to the flow of alternating current of frequency, f, is

$$Z = \sqrt{R^2 + \left(2\pi fL - \frac{1}{2\pi fC}\right)^2}$$

In the resistance box arm of the bridge the calculation is a little more complicated and will be given in greater detail.

The impedance, Z, may be resolved into two components, the resistance, R, and the reactance, X, such that

$$Z = \sqrt{R^2 + X^2}$$

where the reactance

$$X = 2\pi fL - \frac{1}{2\pi fC}$$

The admittance, Y, is the reciprocal of the impedance, Z; the conductance, G, is the reciprocal of the resistance, R; and the susceptance, B, is the reciprocal of the reactance, X, and

$$Y = \sqrt{G^2 + B^2}$$

or for the parallel circuit

$$Z = \frac{1}{Y} = \frac{1}{\sqrt{G^2 + B^2}} = \frac{1}{\sqrt{\left(\frac{R_1}{Z_1^2} + \frac{R_2}{Z_2^2}\right)^2 + \left(\frac{X_1}{Z_1^2} + \frac{X_2}{Z_2^2}\right)^2}}$$

But if the inductance and capacity of the resistance box are disregarded as well as the resistance and inductance of the variable condenser, and if the latter is so constructed to be of negligible dielectric loss, the equation may be simplified and reduced to the following.

$$Z = \frac{1}{\sqrt{\frac{1}{R_1^2} + (-2\pi fC_2)^2}}$$

In case the self-inductance of the conductivity cell is negligible, the resistance, R, of the solution may be found from the following proportion.

$$\sqrt{R^2 + \left(-\frac{1}{2\pi fC}\right)^2} : \frac{1}{\sqrt{\frac{1}{R_1^2} + (-2\pi fC_2)}} = R_3 : R_4$$

Example.—The following determination was made on 1 per cent NaCl in water in a conductivity cell containing bright gold electrodes 25 mm. square, at 25°. $R_1 = 20$ ohms, $C_2 = 1.06 \times 10^{-6}f$, $R_3 = 478$ and $R_4 = 522$, and f = 5,000. The impedance of the arm of the bridge containing resistance box and variable condenser was

$$\frac{1}{\sqrt{\frac{1}{20^2} + (6.2832 \times 5{,}000 \times 1.06 \times 10^{-6})^2}} = 16.67 \text{ ohms}$$

and the impedance of the cell: 16.67 = 478: 522, therefore the impedance of the cell = 15.28.

Since the difference in phase between the current and voltage is the same in the two arms of the bridge, the following ratio holds.

$$\frac{1}{R_x} : \frac{1}{16.67} = R : 15.28$$

therefore $R = 16.67 \times 15.28 \div 20 = 12.73$ ohms

It should be emphasized that if the usual method of disregarding the reactance of the bridge is followed the calculation is

$$R : 20 = 478 : 522$$

therefore

$$R = 18.314$$

in other words, the resistance of the solution is 12.73 ohms, whereas the uncorrected calculation gives 18.314 ohms, or 28 per cent too high a value.

A Method of Procuring Sharp Tone-Minimum with Bright Electrodes without the Necessity of Complicated Calculations.

If the variable condenser, $C_2R_2L_2$, is removed from the Wheatstone bridge, a tone-minimum of great sharpness may be secured with the aid of a variable inductor, L, in series with the con-

ductivity cell. With bright electrodes, 25 mm. square, we found the General Radio Variometer 107 *C*, with a range of about 0.02 to 0.5 millihenries sufficient, and the cost was a small fraction of that of the variable condenser necessitated by the previous method. Since there is loss of power due to dielectric hysteresis in a paraffined paper condenser, a high grade condenser of maximum capacity of 10 microfarads had to be used in the previous method and hence the high cost.

If the reactance of the resistance box is negligible, the reactance of the conductivity cell plus variometer is zero when the variometer is adjusted for sharp tone silence, as follows:

$$\text{Reactance} = 2\pi fL - \frac{1}{2\pi fC}$$

and tone-minimum is not secured until the inductance, L, is so adjusted that it compensates the capacity, *C*, of the cell so that the reactance becomes zero.

With the elimination of the reactance from the equation, the calculation involves only the resistances in the four arms of the bridge and the subtraction of the resistance of the variometer from the resistance of the arm of the bridge containing it, in order to obtain the resistance of the conductivity cell. The variometer is made of two coils rotating on an axis in the plane of the coils, and hence the current passes through the whole length of wire no matter what the setting for inductance. Therefore, the resistance of the variometer does not change except at frequencies high enough to produce skin effect. The resistance of the variometer may be determined once for all. Its resistance was 1.095 with coils connected in series and 0.2855 with coils connected in parallel for low inductance.

The use of the variometer eliminates the necessity of platinizing the electrodes, and allows the use of smaller electrodes, thus adapting the conductivity method to a wider range of usefulness in biochemistry. Sufficient current must be used in order to work the telephone diaphragm, but, if the conductivity of the solution is high enough, very small electrodes may be used without danger of reaching current saturation. An inductance has been used by Taylor and Acree,[1] but their results were reduced to infinite frequency.

[1] Taylor, W. A., and Acree, S. F., *J. Am. Chem. Soc.*, 1916, xxxviii, 2403.

RATE OF HYDROLYSIS OF PHOSPHORIC ESTERS OF SUGAR DERIVATIVES.

First Paper.

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(Received for publication, June 20, 1920.)

A number of the components of the tissues contain in their molecules either phosphoric or sulfuric acid. The phosphoric acid radicle is contained in the mono- and polynucleotides, and the sulfuric acid radicle in chondroitin- and mucoitin-sulfuric acids. In all these compounds the acid radicle is linked in ester form to either a carbohydrate or a polyhydric alcohol. On long acquaintance with substances of this character, one comes to realize that the inorganic radicle is linked in different substances of the same group with different degrees of firmness. Thus, for instance, in inosinic and uridinphosphoric acids on one hand, and in guanylic on the other; in chondroitin-sulfuric on one hand, and in mucoitin sulfuric on the other, the organic acids display different degrees of resistance towards hydrolytic agents.

In compounds such as the ribose nucleotides, two components of the molecule, the sugar, and the inorganic acid, remain constant, and only the third component is variable. It is evident that the firmness of the union between the acid and the rest of the molecule may be conditioned by two factors: the character of the base, and the position of the acid radicle on the sugar molecule.

There exists no systematic study of the rate of hydrolysis of ester form derivatives of sugars which have only one point of difference; namely, that of the allocation of the acid radicle. However, in the preparative work on sugar derivatives by Fischer[1] and his students, and by Irvine[2] and his students, one

[1] Fischer, E., and Noth, H., *Ber. chem. Ges.*, 1918, li, 321.

[2] Irvine, J. C., and Scott, J. P., *J. Chem. Soc.*, 1913, ciii, 575.

finds abundant evidence that such differences in the rate of hydrolysis exist.

A more detailed as well as a more systematic investigation of this problem is much desired not only for purely academic considerations, but also for what, in a way, may be regarded as practical or applied reasons.

It is a difficult task to determine the allocation of an acyl group on the sugar molecule by direct chemical method. On few occasions the method was successful, and only then when the substitution took place on the primary alcoholic group. When any of the secondary alcoholic groups are substituted the allocation of the substituting group has thus far not been successful. As yet the present work does not embrace a sufficiently large number of esters, and is offered in its present form because one of the authors is compelled to discontinue his cooperation.

The substances[3] employed in this work were phosphoric esters of: (1) α-methylglucoside; (2) β-, γ-, ϵ-trimethyl methylglucoside; (3) α-, β-, γ-, ϵ-diacetone glucose; (4) α-, β-monoacetone glucose; (5) a phosphoric ester of the same sugar derivative as in (4), but differing from the substance (4) by the position of the phosphoric acid radicle; (6) ζ-benzoyl-α-β-monoacetone glucose.

In the first, the position of the phosphoric acid radicle is not established, and the interest attached to the substance is due primarily to the fact that the sugar radicle in it is non-substituted. In the following two substances, namely β-, γ-, ϵ-trimethyl methylglucoside and diacetone glucose, the phosphoric acid radicle is attached to the same carbon atom, and hence the two substances differ in the nature and in the molecular weight of the substituting groups. Differences in the rate of hydrolysis of these two substances should be attributed to the influence of these groups.

The fourth substance differed from the fifth in the method of preparation. Whereas the latter was obtained by the action of phosphorous oxychloride on monoacetone glucose, the former is formed as a by-product by the action of phosphorous oxychloride on diacetone glucose. Apparently in the former substance the phosphoric acid radicle was linked to the primary alcoholic group,

[3] The preparation of these substances will be described in a subsequent paper by P. A. Levene and G. M. Meyer.

whereas in the latter it was linked to one of the secondary alcoholic groups.

The sixth substance, ζ-benzoyl-α-β-monoacetone glucosidophosphoric acid, differs from the preceding two by the position of the phosphoric acid radicle, by the number of the substituting groups, and by the difference in character of one of the substituting groups.

The hydrolysis of these substances was carried out in approximately equivalent concentration, which was in the neighborhood of 10 per cent. As catalytic agent 0.1 N sulfuric acid was employed. The temperature of hydrolysis was 100°C.

As was anticipated, the rate of hydrolysis followed the monomolecular law. The constants of hydrolysis $K = \frac{1}{t} \log \frac{a}{a-x}$ were as follows:

$K_1 = 22\ (10^{-3})$
$K_2 = 43\ (10^{-3})$
$K_3 = 56\ (10^{-3})$
$K_4 = 44\ (10^{-3})$
$K_5 = 58\ (10^{-3})$
$K_6 = 18\ (10^{-3})$

(The subscripts indicate the substances in the order of their tabulation.)

Thus in this series of substances apparently both the position of the phosphoric acid radicle and the nature of the substituting groups exert an influence on the stability of the phosphoric ester linking.

On the other hand, the constants of hydrolysis of the sugar derivatives which differ from one another only in point of position of the acid radicle, as Substances 4 and 5, are determined by the position of the inorganic radicle.

It was expected that the position of the phosphoric acid radicle would play the determining part in the rate of hydrolysis. This expectation has not materialized, and it will therefore be necessary to prepare such phosphoric esters of glucose or of methylglucoside which will have the inorganic radicle in a known position, and which will be free from other substituting groups.

It is planned to continue the work in this direction in this laboratory.

EXPERIMENTAL.

α-Methylglucosidophosphoric Acid.—11.644 gm. of barium methylglucosidophosphate were ground up with a little water, 49.2 cc. of N H_2SO_4 were added, and the volume was made up to 100 cc. The solution was filtered from the barium sulfate without further addition of water.

The concentration of methylglucosidophosphoric acid was established by a polariscopic determination; 5 cc. of the solution contained 0.450 gm. of methylglucosidophosphoric acid, representing 0.0509 gm. of P.

5 cc. of this solution were pipetted into glass tubes together with 5 cc. of 0.2 N H_2SO_4. The tubes were sealed and heated in an oil bath at 100°C. for various intervals as indicated in the tables. After cooling, the contents of each tube were made up to 100 cc. and the phosphorous was determined as magnesium pyrophosphate on 40 cc. of this solution.

The results are tabulated in Tables I, I*a*, II, and II*a*.

β-γ-ε-Trimethyl Methylglucosido-ζ-Phosphoric Acid.—13.0785 gm. of the barium salt were ground up in a little water, the barium was precipitated by 42.7 cc. of N H_2SO_4, and the solution made up to 100 cc. 5 cc. of this solution contained 0.45 gm. of trimethyl methylglucosidophosphoric acid, determined polarimetrically, equivalent to 0.0441 gm. of phosphorus.

5 cc. of the filtrate were heated in sealed tubes with 5 cc. of 0.2 N H_2SO_4 for various periods at 100° and the phosphorus was determined as magnesium pyrophosphate.

The results are tabulated in Tables III, III*a*, IV, and IV*a*.

α-β-γ-ε-Diacetone Glucosido-ζ-Phosphoric Acid.—10.1805 gm. of the barium salt were dissolved in a small volume of water and made up to 75 cc. 5 cc. of this solution, containing 0.450 gm. of diacetone glucoside phosphoric acid, equivalent to 0.041 gm of phosphorus, were pipetted into glass tubes together with 3.3 cc. of N H_2SO_4 and 1.7 cc. of water. The tubes were sealed and heated at 100° for various intervals.

The results are tabulated in Tables V and V*a*.

α-β-Monoacetone Glucosido-ζ-Phosphoric Acid (from Diacetone Glucose).—7.5617 gm. of the barium salt of this substance were dissolved in a small volume of warm water and made up to 50 cc.

Of this solution 3 cc., equivalent to 0.029 gm. of P, were pipetted into glass tubes together with 2.27 cc. of N H_2SO_4 and 0.73 cc. of water and sealed. The tubes were heated at 100° for various intervals.

The results are tabulated in Tables VI and VI*a*.

α-β-Monoacetone Glucosidophosphoric Acid (from Monoacetone Glucose).—5.876 gm. of the barium salt of the substance were dissolved in a small quantity of warm water and made up to 40 cc. 3 cc. of this solution, equivalent to 0.028 gm. of P, together with 2.29 cc. of N H_2SO_4 and 0.71 cc. of water were heated in sealed tubes for various intervals.

The results are tabulated in Tables VII and VII*a*.

ζ-Benzoyl-α-β-Monoacetone Glucosidophosphoric Acid.—8.4285 gm. of the barium salt of the substance were rubbed up with a small volume of warm water, made up to 75 cc., and filtered. 5 cc. of the filtrate, equivalent to 0.035 gm. of P, together with 2.97 cc. of N H_2SO_4 and 2.03 cc. of water were heated in sealed tubes for various intervals.

The results are tabulated in Tables VIII and VIII*a*.

TABLE I.

α-Methylglucosidophosphoric Acid.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0015	0.0016	0.0004	0.24	2.16
	0.0016				
	0.0017	0.0017	0.0005	0.27	2.36
	0.0016				
2	0.0043	0.0044	0.0012	0.69	6.09
	0.0045				
	0.0044	0.0045	0.0013	0.69	6.09
	0.0045				
4	0.0081	0.0082	0.0023	1.27	11.20
	0.0083				
	0.0085	0.0087	0.0024	1.34	11.98
	0.0088				
8	0.0175	0.0173	0.0048	2.69	23.77
	0.0171				
	0.0177	0.0178	0.0050	2.76	24.36
	0.0178				
16	0.0296	0.0294	0.0082	4.56	40.27
	0.0292				
	0.0304	0.0299	0.0083	4.62	40.86
	0.0294				
24	0.0394	0.0395	0.0110	6.11	54.03
	0.0396				
	0.0396	0.0397	0.0111	6.11	54.03
	0.0398				

TABLE II.

α-Methylglucosidophosphoric Acid.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0014	0.0015	0.0004	0.24	2.16
	0.0016				
	0.0018	0.0017	0.0005	0.27	2.36
	0.0016				
2	0.0044	0.0043	0.0012	0.67	5.89
	0.0042				
	0.0042	0.0044	0.0012	0.69	6.09
	0.0046				
4	0.0082	0.0082	0.0023	1.27	11.20
	0.0081				
	0.0087	0.0087	0.0024	1.33	11.79
	0.0086				
8	0.0170	0.0170	0.0047	2.62	23.18
	0.0170				
	0.0170	0.0170	0.0047	2.62	23.18
	0.0170				
16	0.0304	0.0306	0.0085	4.78	42.24
	0.0308				
	0.0314	0.0315	0.0088	4.89	43.22
	0.0315				
24	0.0409	0.0408	0.0114	6.31	55.78
	0.0406				
	0.0417	0.0418	0.0116	6.47	57.17
	0.0418				
32	0.0504	0.0504	0.0140	7.80	68.96
	0.0504				
	0.0500	0.0501	0.0140	7.76	68.56
	0.0502				

TABLE III.

β-γ-ε-Trimethyl Methylglucosido-ζ-Phosphoric Acid.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0025	0.0025	0.0007	0.38	3.83
	0.0025				
	0.0026	0.0026	0.0007	0.40	4.05
	0.0026				
2	0.0071	0.0070	0.0020	1.09	11.04
	0.0069				
	0.0060	0.0068	0.0019	1.04	10.59
	0.0068				
4	0.0124	0.0125	0.0035	1.93	19.60
	0.0125				
	0.0129	0.0125	0.0035	1.93	19.60
	0.0120				
8	0.0247	0.0247	0.0069	3.82	38.74
	0.0247				
	0.0242	0.0241	0.0067	3.73	37.84
	0.0240				
16	0.0403	0.0403	0.0112	6.22	63.06
	0.0402				
	0.0410	0.0411	0.0115	6.36	64.42
	0.0412				
24	0.0527	0.0527	0.0147	8.16	82.66
	0.0526				
	0.0524	0.0526	0.0147	8.13	82.43
	0.0527				

TABLE IV.

β-γ-ε-Trimethyl Methylglucosido-ζ-Phosphoric Acid.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0028 0.0026	0.0027	0.0008	0.42	4.24
	0.0026 0.0025	0.0026	0.0007	0.40	4.08
2	0.0071 0.0074	0.0073	0.0020	1.13	11.46
	0.0068 0.0070	0.0069	0.0019	1.07	10.83
4	0.0130 0.0130	0.0130	0.0036	2.01	20.40
	0.0125 0.0130	0.0128	0.0036	1.98	20.09
8	0.0245 0.0246	0.0246	0.0069	3.81	38.60
	0.0246 0.0245	0.0245	0.0068	3.79	38.45
16	0.0388 0.0390	0.0389	0.0108	6.02	61.04
	0.0386 0.0390	0.0388	0.0108	6.00	60.81
24	0.0496 0.0492	0.0494	0.0138	7.64	77.48
	0.0492 0.0494	0.0493	0.0137	7.62	77.25

TABLE V.

α-β-γ-ε-Diacetone Glucoside-ζ-Phosphoric Acid.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0040	0.0040	0.0011	0.62	6.80
	0.0040				
	0.0037	0.0039	0.0011	0.62	6.80
	0.0040				
2	0.0090	0.0088	0.0025	1.36	14.95
	0.0085				
	0.0091	0.0091	0.0025	1.41	15.46
	0.0090				
4	0.0159	0.0157	0.0044	2.43	26.68
	0.0157				
	0.0154	0.0156	0.0043	2.42	26.51
	0.0158				
8	0.0262	0.0262	0.0073	4.06	44.52
	0.0262				
	0.0262	0.0262	0.0073	4.06	44.52
	0.0262				
16	0.0419	0.0429	0.0117	6.64	72.90
	0.0438				
	0.0419	0.0419	0.0117	6.49	71.20
	0.0394				
24	0.0525	0.0525	0.0146	8.13	89.22
	0.0525				
	0.0525	0.0525	0.0146	8.13	89.22
	0.0525				

TABLE VI.

α-β-Monoacetone Glucosido-ζ-Phosphoric Acid (from Diacetone Glucose).

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0050	0.0049	0.0014	0.78	7.56
	0.0047				
	0.0048	0.0048	0.0014	0.78	7.56
	0.0047				
2	0.0103	0.0100	0.0028	1.56	15.12
	0.0097				
	0.0100	0.0100	0.0028	1.56	15.12
	0.0100				
4	0.0183	0.0182	0.0051	2.84	27.65
	0.0180				
	0.0175	0.0174	0.0048	2.67	25.92
	0.0173				
8	0.0323	0.0323	0.0090	5.00	48.60
	0.0323				
	0.0320	0.0320	0.0089	4.96	48.16
	0.0320				
16	0.0483	0.0483	0.0135	7.51	73.00
	0.0483				
	.....	0.0483	0.0135	7.51	73.00
	0.0483				
24	0.0570	0.0570	0.0159	8.84	85.96
	0.0570				
	0.0570	0.0570	0.0159	8.84	85.96
	0.0570				

TABLE VII.

α-β-Monoacetone Glucosidophosphoric Acid (from Monoacetone Glucose).

Time	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0033	0.0036	0.0010	0.55	5.40
	0.0038				
	0.0035	0.0036	0.0010	0.55	5.40
	0.0037				
2	0.0073	0.0077	0.0021	1.18	11.45
	0.0080				
	0.0075	0.0076	0.0021	1.18	11.45
	0.0077				
4	0.0150	0.0152	0.0042	2.33	22.68
	0.0153				
	0.0143	0.0144	0.0040	2.22	21.60
	0.0145				
8	0.0260	0.0265	0.0074	4.11	39.96
	0.0270				
	0.0268	0.0265	0.0074	4.11	39.96
	0.0261				
16	0.0405	0.0407	0.0113	6.29	61.12
	0.0408				
	0.0403	0.0403	0.0112	6.22	60.47
	0.0403				
24	0.0511	0.0511	0.0142	7.89	76.67
	0.0511				
	0.0508	0.0509	0.0142	7.89	76.67
	0.0510				

TABLE VIII.

ζ-Benzoyl-α-β-Monoacetone Glucosidophosphoric Acid.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0012	0.0012	0.0003	0.19	2.42
	0.0012				
	0.0012	0.0012	0.0003	0.19	2.42
	0.0012				
2	0.0022	0.0022	0.0006	3.40	4.42
	0.0022				
	0.0021	0.0021	0.0006	3.24	4.42
	0.0021				
4	0.0048	0.0048	0.0013	7.42	9.65
	0.0048				
	0.0048	0.0048	0.0013	7.42	9.65
	0.0048				
8	0.0094	0.0094	0.0026	14.56	18.93
	0.0094				
	0.0094	0.0094	0.0026	14.56	18.93
	0.0094				
16	0.0174	0.0174	0.0048	26.93	35.03
	0.0173				
	0.0174	0.0174	0.0048	26.93	35.03
	0.0174				
24	0.0223	0.0223	0.0062	34.53	44.92
	0.0223				
	0.0223	0.0223	0.0062	34.53	44.92
	0.0223				

Velocity Constants.

TABLE Ia.

α-Methylglucosidophosphoric Acid.

t	$Mg_2P_2O_7$ (x)	$a - x$	$\frac{1}{t} \log \frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0017	0.0714	0.00017
120	0.0045	0.0686	0.00023
240	0.0085	0.0646	0.00022
480	0.0176	0.0555	0.00025
960	0.0297	0.0434	0.00023
1,440	0.0396	0.0335	0.00023
	$a = 0.0731$		
Average.....			0.00022

TABLE IIa.

α-Methylglucosidophosphoric Acid.

t	$Mg_2P_2O_7$ (x)	$a - x$	$\frac{1}{t} \log \frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0016	0.0715	0.00016
120	0.0044	0.0687	0.00022
240	0.0085	0.0646	0.00022
480	0.0170	0.0561	0.00023
960	0.0311	0.0420	0.00025
1,440	0.0413	0.0318	0.00025
1,920	0.0503	0.0228	0.00026
	$a = 0.0731$		
Average.....			0.00022

TABLE IIIa.

β-γ-ε-Trimethyl Methylglucosido-ζ-Phosphoric Acid.

t	$Mg_2P_2O_7$ (x)	$a - x$	$\frac{1}{t} \log \frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0026	0.0608	0.00030
120	0.0069	0.0565	0.00042
240	0.0125	0.0509	0.00040
480	0.0244	0.0390	0.00044
960	0.0407	0.0227	0.00046
1,440	0.0527	0.0107	0.00053
	$a = 0.0634$		
Average			0.00044

TABLE IVa.

β-γ-ε-Trimethyl Methylglucosido-ζ-Phosphoric Acid.

t	$Mg_2P_2O_7$ (x)	$a - x$	$\frac{1}{t} \log \frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0027	0.0607	0.00032
120	0.0071	0.0563	0.00043
240	0.0129	0.0505	0.00041
480	0.0246	0.0388	0.00044
960	0.0389	0.0245	0.00043
1,440	0.0494	0.0140	0.00045
	$a = 0.0634$		
Average			0.00041

TABLE Va.

α-β-γ-ε-Diacetone Glucosido-ζ-Phosphoric Acid.

t	$Mg_2P_2O_7$ (x)	$a - x$	$\frac{1}{t} \log \frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0040	0.0549	0.00050
120	0.0090	0.0499	0.00060
240	0.0157	0.0432	0.00056
480	0.0262	0.0327	0.00053
960	0.0424	0.0165	0.00058
1,440	0.0525	0.0064	0.00067
	$a = 0.0589$		
Average			0.00056

TABLE VIa.

α-β-Monoacetone Glucose-ζ-Phosphoric Acid (from Diacetone Glucose).

t	$Mg_2P_2O_7(x)$	$a - x$	$\frac{1}{t} \log \frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0049	0.06168	0.000553
120	0.0100	0.05658	0.000588
240	0.0178	0.04878	0.000563
480	0.0322	0.03438	0.000598
960	0.0483	0.01828	0.000585
1,440	0.0570	0.00958	0.000585
	$a = 0.06658$		
Average			0.000579

TABLE VIIa.

α-β-Monoacetone Glucosidophosphoric Acid (from Monoacetone Glucose).

t	$Mg_2P_2O_7(x)$	$a - x$	$\frac{1}{t} \log \frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0036	0.06298	0.000400
120	0.0077	0.05888	0.000445
240	0.0148	0.05178	0.000455
480	0.0265	0.04008	0.000459
960	0.0405	0.02608	0.000423
1,440	0.0510	0.01558	0.000438
	$a = 0.06658$		
Average			0.000437

TABLE VIIIa.

ζ-Benzoyl-α-β-Monoacetone Glucosidophosphoric Acid.

t	$Mg_2P_2O_7(x)$	$a - x$	$\frac{1}{t} \log \frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0012	0.0485	0.000177
120	0.0022	0.0475	0.000164
240	0.0048	0.0449	0.000183
480	0.0094	0.0403	0.000189
960	0.0174	0.0323	0.000194
1,440	0.0223	0.0274	0.000180
	$a = 0.0497$		
Average			0.000181

HYDROLYSIS OF NUCLEOTIDES.

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Early in the work on nucleotides it was observed that individual members of this group differed in their resistance to the action of hydrolytic agents. Owing to this peculiarity, Levene and Mandel obtained on hydrolysis of thymus nucleic acid a mononucleotide: thymidinphosphoric acid.[1] Later, Levene, and Levene and Jacobs[2] prepared the pyrimidin nucleotides of yeast nucleic acid and Levene and Jacobs,[3] on hydrolysis of thymus nucleic acid, prepared hexocytidin- and hexothymidindiphosphoric acids. Jones[4] and his coworkers furnished further observations demonstrating this peculiarity of different nucleotides.

The structural details of the molecule which are responsible for this difference in the behavior of the individual mononucleotides are as yet unknown.

Referring only to the ribose nucleotides, the observations are as follows: Inosinic, uridinphosphoric, and cytidinphosphoric acids demonstrated a higher resistance, whereas adenosinphosphoric and guanosinphosphoric acids possess a lower resistance towards hydrolytic agents.

It is certain that all these nucleotides differ from one another in the nature of the base, but it is also possible that they differ in the position of the phosphoric acid radicle on the carbohydrate.

In only one of these nucleotides, in inosinic acid, is the position of the phosphoric acid known; namely, it is linked to the fifth carbon atom. Whether the stability of this substance is condi-

[1] Levene, P. A., and Mandel, J. A., *Ber. chem. Ges.*, 1908, xli, 1905.

[2] Levene, P. A., *Biochem. Z.*, 1909, xvii, 120. Levene, P. A., and Jacobs, W., *Ber. chem. Ges.*, 1911, xliv, 1027.

[3] Levene, P. A., and Jacobs, W., *J. Biol. Chem.*, 1912, xii, 411.

[4] Jones, W., and Read, B. E., *J. Biol. Chem.*, 1917, xxix, 123.

tioned by the position of the phosphoric acid radicle cannot as yet be stated. There are indications that this may be so, since the ribose phosphoric acid obtained from this nucleotide also possesses considerable stability.

The differences in the stability hitherto observed are only of a qualitative nature. However, in order that this property of nucleotides may be made the basis for suggestions regarding their structure, it is necessary to obtain more detailed information concerning the comparative rate of hydrolysis of the various nucleotides.

The substances employed in this investigation were as follows: Yeast nucleic acid (employed in form of its barium salt), inosinic acid (in form of barium salt), uridinphosphoric acid (barium salt), guanylic acid (crystalline nucleotide), adenylic acid (crystalline nucleotide), and hexothymidindiphosphoric acid (barium salt).

These substances were employed in approximately equivalent concentrations, and the hydrolysis was accomplished by 0.1 N sulfuric acid at a temperature of 100°C. It was found by Levene and the writer that consistent results were obtained only when the hydrolysis was carried out in sealed tubes. The rate of hydrolysis followed, as was expected, the monomolecular law and the constant of hydrolysis $K = \frac{1}{t} \log \frac{a}{a-x}$ was as follows:

	$\frac{1}{t} \log \frac{a}{a-x}$
Nucleic acid	130 (10^{-2})
Guanosinphosphoric acid	177 (10^{-2})
Adenosinphosphoric "	166 (10^{-2})
Uridinphosphoric "	480 (10^{-3})
Inosinic acid	470 (10^{-3})
Hexothymidindiphosphoric acid	726 (10^{-3})

Thus the velocity constants of the guanosinphosphoric and adenosinphosphoric acids is identical. On the other hand the constants of inosinic and uridinphosphoric are also identical in spite of the fact that their basic component is of a different nature.

EXPERIMENTAL.

Nucleic Acid.—4.1184 gm. of barium nucleate were ground up in a small quantity of water and 16.3 cc. of N H_2SO_4, made up to 30 cc., and filtered. 5 cc. of this solution by Kjeldahl determination contained 0.4184 gm. of nucleic acid equivalent to 0.0397 gm. of phosphorus.

5 cc. of this solution were hydrolyzed with 5 cc. of 0.2 N H_2SO_4 in sealed tubes at 100° for various intervals. The phosphorus was estimated as magnesium pyrophosphate. The results are tabulated in Tables I and I*a*.

In Series 2 and 3, 0.5607 gm. of barium nucleate was weighed off for each tube, equivalent to 0.450 gm. of nucleic acid, and this was hydrolyzed with 1.61 cc. of N H_2SO_4, and 3.39 cc. of water. The contents of these tubes after heating were treated as previously described. The results are tabulated in Tables II, II*a*, III, and III*a*.

Guanosinphosphoric Acid.—6.75 gm. of guanylic acid were dissolved in a small quantity of warm water and made up to 75 cc. By analysis of a sample of this material each 5 cc. of the above solution contained 0.4321 gm. of guanylic acid or an equivalent of 0.0365 gm. of phosphorus. 5 cc. of the guanylic acid solution were hydrolyzed with 5 cc. of 0.2 N H_2SO_4 at 100° for various intervals.

The results are tabulated in Tables IV and IV*a*.

Adenosinphosphoric Acid.—4.733 gm. of adenylic acid are dissolved in a small quantity of warm water and made up to 50 cc.

3 cc. of this solution equivalent to 0.024 gm. of P and 3 cc. of 0.2 N H_2SO_4 are hydrolyzed in sealed tubes at 100° for various intervals.

The results are tabulated in Tables V and V*a*.

Uridinphosphoric Acid.—Series I.—10.5404 gm. of barium uridinphosphate were taken up in water and 44.3 cc. of N H_2SO_4. The solution was filtered and 5 cc. of the filtrate containing 0.450 gm. of uridinphosphoric acid (Kjeldahl) equivalent to 0.044 gm. of P are hydrolyzed with 5 cc. of 0.2 N H_2SO_4 at 100°C. for various intervals. The results are tabulated in Tables VI and VI*a*.

Series II.—9.6435 gm. of barium uridinphosphate are taken up in a little water and 41.6 cc. of N H_2SO_4 and further treated

as in the previous series. Results are tabulated in Tables VII and VII*a*.

Inosinphosphoric Acid.—11.9936 gm. of barium inosinate were dissolved in a small quantity of water and the barium was precipitated with 38.2 cc. of N H_2SO_4. The solution was made up to 75 cc. and filtered. An analysis of the solution (Kjeldahl) showed 5 cc. of solution to contain 0.4453 gm. of inosinic acid equivalent to 0.0397 gm. of phosphorus.

5 cc. of the filtrate were hydrolyzed with 5 cc. of 0.2 N H_2SO_4 at 100°C. for various periods. The results are tabulated in Tables VIII and VIII*a*.

Hexothymidindiphosphoric Acid.—9.893 gm. of the barium salt were ground up in a little water and 24.4 cc. of 2 N H_2SO_4 and the volume was made up to 50 cc. The solution was filtered and of the filtrate 3 cc. were hydrolyzed with 3 cc. of 0.2 N H_2SO_4 at 100°C. for various intervals. The concentration of the material was established by means of a Kjeldahl nitrogen determination.

3 cc. of the filtrate were equivalent to 0.03947 gm. of phosphorus. The results are tabulated in Tables IX and IX*a*.

TABLE I.

Nucleic Acid.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0089 0.0088	0.0089	0.0025	1.48	15.62
	0.0090 0.0088	0.0089	0.0025	1.48	15.62
4	0.0308 0.0306	0.0307	0.0086	5.11	53.90
	0.0309 0.0310	0.0310	0.0086	5.16	54.41

TABLE II.

Nucleic Acid.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0062	0.0062	0.0017	0.94	14.19
	0.0062				
	0.0062	0.0062	0.0017	0.94	14.19
	0.0077				
2	0.0135	0.0135	0.0038	2.11	31.73
	0.0135				
	0.0149	0.0144	0.0040	2.22	33.40
	0.0144				
4	0.0251	0.0250	0.0070	3.89	58.44
	0.0248				
	0.0253	0.0254	0.0071	3.94	59.28
	0.0254				
8	0.0363	0.0360	0.0100	5.56	83.49
	0.0357				
	0.0368	0.0369	0.0103	5.72	85.99
	0.0370				
16	0.0437	0.0436	0.0122	6.78	101.86
	0.0434				
	0.0440	0.0440	0.0123	6.78	101.86
	0.0440				
24	0.0458	0.0459	0.0128	7.11	106.87
	0.0459				
	0.0467	0.0465	0.0130	7.22	108.54
	0.0462				

TABLE III.

Nucleic Acid.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0057	0.0058	0.0016	0.89	13.36
	0.0059				
	0.0059	0.0060	0.0017	0.94	14.19
	0.0061				
2	0.0133	0.0135	0.0038	2.11	31.73
	0.0136				
	0.0132	0.0131	0.0037	2.06	30.89
	0.0130				
4	.....	0.0252	0.0070	3.89	58.44
	0.0252				
	0.0254	0.0255	0.0071	3.94	59.28
	0.0256				
8	0.0364	0.0364	0.0101	5.56	83.49
	0.0364				
	0.0366	0.0369	0.0103	5.72	85.99
	0.0372				
16	0.0438	0.0438	0.0122	6.78	101.86
	0.0438				
	0.0436	0.0435	0.0121	6.72	101.02
	0.0434				

TABLE IV.

Guanosinphosphoric Acid.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0098	0.0098	0.0027	1.57	18.43
	0.0098				
	0.0096	0.0096	0.0027	1.55	18.16
	0.0096				
2	0.0200	0.0200	0.0056	3.22	37.67
	0.0201				
	0.0198	0.0198	0.0055	3.19	37.40
	.....				
4	0.0338	0.0341	0.0095	5.51	64.50
	0.0344				
	0.0337	0.0337	0.0094	5.44	63.67
	0.0336				
8	0.0449	0.0450	0.0125	7.27	85.09
	0.0451				
	0.0447	0.0448	0.0125	7.22	84.55
	0.0449				
12	0.0490	0.0488	0.0136	7.87	92.14
	0.0485				
	0.0490	0.0488	0.0136	7.87	92.14
	0.0485				
16	0.0495	0.0496	0.0138	8.01	93.77
	0.0497				
	0.0493	0.0493	0.0137	7.94	92.95
	0.0493				
24	0.0507	0.0507	0.0141	8.19	95.94
	0.0507				
	0.0504	0.0505	0.0141	8.15	95.39
	0.0505				

TABLE V.

Adenosinphosphoric Acid.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0110	0.0110	0.0031	1.71	19.15
	0.0110				
	0.0110	0.0110	0.0031	1.71	19.15
	0.0110				
2	0.0223	0.0223	0.0062	3.44	38.56
	0.0223				
	0.0218	0.0224	0.0062	3.44	38.56
	0.0230				
4	0.0372	0.0371	0.0103	5.73	64.18
	0.0370				
	0.0375	0.0375	0.0105	5.84	65.42
	0.0375				
8	0.0475	0.0477	0.0133	7.40	82.84
	0.0478				
	0.0482	0.0479	0.0133	7.40	82.84
	0.0475				
16	0.0537	0.0537	0.0150	8.33	93.28
	0.0537				
	0.0535	0.0536	0.0149	8.29	92.79
	0.0537				
24	0.0540	0.0544	0.0152	8.44	94.53
	0.0547				
	0.0538	0.0543	0.0152	8.44	94.53
	0.0548				

TABLE VI.

Uridinphosphoric Acid.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0030	0.0030	0.0008	0.45	4.75
	0.0030				
	0.0029	0.0030	0.0008	0.45	4.75
	0.0030				
2	0.0074	0.0074	0.0021	1.13	11.76
	0.0074				
	.....	0.0074	0.0021	1.13	11.76
	0.0074				
4	0.0120	0.0136	0.0038	2.06	21.49
	0.0136				
	0.0134	0.0136	0.0038	2.06	21.49
	0.0137				
8	0.0257	0.0255	0.0071	3.85	40.27
	0.0253				
	0.0257	0.0260	0.0072	3.92	40.95
	0.0263				
16	0.0415	0.0415	0.0116	6.26	65.38
	0.0415				
	0.0417	0.0419	0.0117	6.32	66.06
	0.0421				
24	0.0512	0.0512	0.0143	7.73	80.77
	0.0512				
	0.0513	0.0513	0.0143	7.73	80.77
	0.0513				

TABLE VII.

Uridinphosphoric Acid.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0034 0.0034	0.0034	0.0009	0.57	5.91
	0.0036 0.0036	0.0036	0.0010	0.59	6.16
2	0.0070	0.0070	0.0020	1.16	12.07
	0.0076 0.0074	0.0075	0.0021	1.23	12.81
4	0.0136	0.0136	0.0038	2.24	23.40
	0.0138 0.0137	0.0138	0.0038	2.26	23.64
8	0.0236	0.0236	0.0066	3.87	40.39
	0.0256 0.0238	0.0247	0.0066	3.89	40.64
16	..... 0.0407	0.0407	0.0113	6.70	69.95
	0.0413 0.0410	0.0412	0.0115	6.77	70.69

TABLE VIII.

Inosinphosphoric Acid.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0033	0.0033	0.0009	0.52	5.79
	0.0033				
	0.0035	0.0035	0.0010	0.55	6.05
	0.0035				
2	0.0059	0.0060	0.0017	0.94	10.53
	0.0060				
	0.0062	0.0062	0.0017	0.97	10.88
	0.0062				
4	0.0135	0.0134	0.0037	2.08	23.42
	0.0132				
	0.0139	0.0138	0.0038	2.16	24.18
	0.0137				
8	0.0219	0.0220	0.0061	3.44	38.54
	0.0220				
	0.0215	0.0213	0.0059	3.32	37.28
	0.0210				
16	0.0362	0.0362	0.0101	5.66	63.48
	0.0362				
	0.0365	0.0364	0.0101	5.70	63.98
	0.0363				
24	0.0463	0.0460	0.0128	7.21	80.86
	0.0457				
	0.0457	0.0458	0.0128	7.16	80.35
	0.0459				

TABLE IX.

Hexothymidindiphosphoric Acid.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0054	0.0053	0.0015	1.37	9.37
	0.0052				
	0.0051	0.0053	0.0015	1.37	9.37
	0.0055				
2	.....	0.0104	0.0029	2.67	18.23
	0.0104				
	0.0103	0.0104	0.0029	2.67	18.23
	0.0104				
4	0.0189	0.0185	0.0052	4.78	32.66
	0.0181				
	0.0183	0.0185	0.0052	4.78	32.66
	0.0186				
8	0.0314	0.0313	0.0087	8.07	55.19
	0.0312				
	0.0313	0.0315	0.0088	8.11	55.44
	0.0316				
16	0.0454	0.0452	0.0126	11.67	79.75
	0.0450				
	0.0456	0.0456	0.0127	11.78	80.51
	0.0456				
24	0.0523	0.0524	0.0146	13.52	92.40
	0.0524				
	0.0520	0.0522	0.0145	13.48	92.15
	0.0524				

Velocity Constants.

TABLE Ia.
Nucleic Acid.

t	$Mg_2P_2O_7$ (x)	$a-x$	$\frac{1}{t}\log\frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0089	0.0481	0.0012
240	0.0308	0.0262	0.0014
	$a = 0.0570$		
Average			0.0013

TABLE IIa.
Nucleic Acid.

t	$Mg_2P_2O_7$ (x)	$a-x$	$\frac{1}{t}\log\frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0062	0.0367	0.0011
120	0.0140	0.0289	0.0014
240	0.0252	0.0177	0.0016
480	0.0365	0.0064	0.0017
960	0.0438		
1,440	0.0462		
	$a = 0.0429$		
Average			0.0014

TABLE IIIa.
Nucleic Acid.

t	$Mg_2P_2O_7$ (x)	$a-x$	$\frac{1}{t}\log\frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0059	0.0370	0.0011
120	0.0131	0.0298	0.0013
240	0.0254	0.0175	0.0016
480	0.0367	0.0062	0.0017
960	0.0437		
	$a = 0.0429$		
Average			0.0013

TABLE IVa.

Guanosinphosphoric Acid.

t	$Mg_2P_2O_7$ (x)	$a - x$	$\frac{1}{t} \log \frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0097	0.0433	0.00146
120	0.0199	0.0331	0.00170
240	0.0339	0.0191	0.00185
480	0.0449	0.0081	0.00170
720	0.0488	0.0042	0.00140
960	0.0495	0.0035	0.00188
	$a = 0.0530$		
Average			0.00167

TABLE Va.

Adenosinphosphoric Acid.

t	$Mg_2P_2O_7$ (x)	$a - x$	$\frac{1}{t} \log \frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0110	0.04674	0.00153
120	0.0224	0.03534	0.00178
240	0.0373	0.02044	0.00188
480	0.0478	0.00994	0.00159
960	0.0537	0.00404	0.00162
1,440	0.0543	0.00344	0.00156
	$a = 0.05774$		
Average			0.00166

TABLE VIa.

Uridinphosphoric Acid.

t	$Mg_2P_2O_7$ (x)	$a - x$	$\frac{1}{t} \log \frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0030	0.0605	0.00035
120	0.0074	0.0561	0.00044
240	0.0136	0.0499	0.00044
480	0.0258	0.0377	0.00047
960	0.0417	0.0218	0.00048
1,440	0.0513	0.0122	0.00049
	$a = 0.0635$		
Average			0.00044

TABLE VII*a*.

Uridinphosphoric Acid.

t	$Mg_2P_2O_7$	$a-x$	$\frac{1}{t}\log\frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0035	0.0549	0.00045
120	0.0073	0.0511	0.00048
240	0.0137	0.0447	0.00048
480	0.0237	0.0347	0.00047
960	0.0410	0.0174	0.00054
	$a = 0.0584$		
Average.....			0.00048

TABLE VIII*a*.

Inosinphosphoric Acid.

t	$Mg_2P_2O_7$ (x)	$a-x$	$\frac{1}{t}\log\frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0034	0.0536	0.00044
120	0.0061	0.0509	0.00041
240	0.0136	0.0434	0.00049
480	0.0217	0.0353	0.00043
960	0.0363	0.0207	0.00046
1,440	0.0459	0.0111	0.00049
	$a = 0.0570$		
Average.....			0.00047

TABLE IX*a*.

Hexothymidindiphosphoric Acid.

t	$Mg_2P_2O_7$ (x)	$a-x$	$\frac{1}{t}\log\frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0053	0.05142	0.000710
120	0.0104	0.04632	0.000733
240	0.0185	0.03822	0.000714
480	0.0314	0.02532	0.000730
960	0.0454	0.01132	0.000717
1,440	0.0522	0.00442	0.000752
	$a = 0.5672$		
Average.....			0.000726

THE ESTIMATION OF AMINOETHANOL AND OF CHOLINE APPEARING ON HYDROLYSIS OF PHOSPHATIDES.

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The method herein described is a modification of the one introduced by Thierfelder and Schulze.[1]

In the original method, the separation of the two bases is based on the difference in the behavior of their hydrochlorides towards calcium oxide. Whereas the hydrochloride of aminoethanol is transformed by calcium oxide into the free base, the salt of choline remains unchanged.

In this particular the present method follows the directions of Thierfelder and Schulze. The difference is in the following.

The extraction of the free aminoethanol is accomplished in the original method by means of ether in a Soxhlet apparatus. This process is time-consuming and presents all the inconveniences of a Soxhlet extraction. We substituted in its stead extraction by means of boiling acetone, which is accomplished very rapidly.

A modification was also introduced for the isolation of choline. In the original process the hydrochloride of the base is extracted with alcohol. From this solution the base is precipitated with mercuric chloride, and this is then transformed into the hydrochloride and as such weighed and identified. However, the hydrochloride does not possess sufficiently desirable properties to encourage its use for the purpose of identification or of quantitative estimation. Hence the method was modified as follows:

The residue from acetone extraction is freed from hydrochloric acid and from lime and the choline is converted into the picrate. This if desired can be converted into the chloroplatinate.

[1] Thierfelder, H., and Schulze, O., *Z. physiol. Chem.*, 1916, xcvi, 296.

EXPERIMENTAL.

The ether-soluble, acetone-insoluble lipoids from egg were freed from white matter, and hydrolyzed by boiling with 3 per cent sulfuric acid for 8 hours as recommended by MacLean.[2] After removal of the fatty acids by filtration, and the sulfuric acid by precipitation with barium hydroxide, the slightly acid filtrate was concentrated in vacuum, and precipitated with basic lead acetate as long as precipitate formed. The precipitate was filtered off, and the filtrate freed from lead with hydrogen sulfide. After addition of hydrochloric acid, the solution of the bases was repeatedly evaporated with water to remove the acetic acid. The residue was then extracted with absolute alcohol, filtered from inorganic salts, and evaporated. The latter operation was repeated twice. Finally the bases were taken up in water and made up to 25 cc. The solution contained 0.887 gm. of total nitrogen and 0.1836 gm. of amino nitrogen.

Of the above solution, 15 cc. were evaporated on the water bath to a small volume. The syrup was thoroughly mixed with an excess of calcium oxide, and extracted three times with dry acetone by boiling it for a few minutes with 75 cc. of that solvent. The combined filtered acetone extracts were acidified with hydrochloric acid, and evaporated in vacuum. The residue was taken up in water and evaporated to a syrup. The treatment with calcium hydroxide and extraction with smaller (25 cc.) portions of acetone were repeated. The final acetone extracts were allowed to stand in an ice chest and filtered. After acidification with hydrochloric acid, the acetone was removed by evaporation in vacuum, and the residue was made up to 10 cc. with water. The solution contained 0.0865 gm. of total nitrogen and 0.08535 gm. of amino nitrogen. Thus 77.5 per cent of the amino nitrogen had been extracted.

Of the above solution 9 cc. were strongly acidified with hydrochloric acid after which 1.9 gm. of gold chloride were added. On standing in a desiccator over sulfuric acid, large crystals separated. These were filtered off, pressed between filter paper, and allowed to dry. The yield was 1.5675 gm. or 71.3 per cent of the theoretical. The substance melted at 188–190°C., and analyzed as follows:

[2] MacLean, H., *Biochem. J.*, 1915, ix, 364.

0.1647 gm. of substance gave 0.0802 gm. of gold on ignition to constant weight.

	Calculated for $C_5H_{13}ON.HAuCl_4$.	Found.
	per cent	*per cent*
Au	49.17	49.06

When the above compound was recrystallized from dilute hydrochloric acid, it separated as large glistening crystals with smooth surface, softening at 185° and melting at 190–192° (uncorrected). The recrystallized compound analyzed as follows:

0.1010 gm. of substance gave 0.0493 gm. of gold on ignition to constant weight.

	Calculated for $C_5H_{13}ON.HAuCl_4$.	Found.
	per cent	*per cent*
Au	49.17	49.11

The residue from the acetone extract was treated with water and filtered from calcium hydroxide. The hydrochloric acid and the remaining calcium were removed from the solution with silver oxide and carbon dioxide, respectively. The filtrate, freed from hydrochloric acid and silver, was acidified with an alcoholic solution of picric acid, concentrated in vacuum, and allowed to stand in an ice chest. The precipitate was filtered off, and recrystallized from water. Beautiful long needles of choline picrate, softening at 238° and melting at 241–242°C. (uncorrected) were thus obtained. The substance analyzed as follows:

0.1302 gm. was reduced with 2 gm. of zinc dust in the presence of 10 cc. of HCl and a little water. The whole was then digested with sulfuric acid as usual, and distilled after addition of enough NaOH to bring all the zinc into solution. The distillate required for titration 15.57 cc. of 0.1 N acid.

0.4164 gm. of the picrate was extracted with ether in the presence of hydrochloric acid. The ether solution was dried over anhydrous sodium sulfate, filtered, and evaporated. The residue was dissolved in dry ether, filtered, and again evaporated. The residue weighed 0.291 gm.

	Calculated for $C_5H_{14}ON.C_6H_2O_7N_3$.	Found.
	per cent	*per cent*
N	16.86	16.75
Picric acid	68.97	69.80

0.7714 gm. of the picrate was extracted with ether as above. The aqueous solution was evaporated and the residue was taken

up in absolute alcohol and precipitated with an alcoholic solution of $PtCl_4$. The yield of dried precipitate was 0.7065 gm. or 98.7 per cent of the theory. The chloroplatinate decomposed at 243°C. and had the following composition.

0.1050 gm. of substance gave on ignition to constant weight 0.0334 gm. of platinum.

	Calculated for $(C_2H_4ONCl)_2PtCl_4$.	Found.
	per cent	*per cent*
Pt	31.64	31.80

UNSATURATED LIPOIDS OF THE LIVER.

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The subject of the lipoids of the liver calls for reinvestigation for several reasons. Previous investigators have laid claim to the discovery in this organ of substances which were not found among the lipoids of other organs. Furthermore, none of the phosphatides isolated from this organ has been identified with sufficient rigor.

The substances described as peculiar to liver tissue are jecorin,[1] and heparphosphatide.[2] Other unsaturated lipoids isolated from the liver are lecithin (Baskoff) and cephalin.[3] The relation of the latter two lipoids to lecithin and to cephalin of other organs had not been established.

Regarding the unsaturated lipoids of other organs it is known that on the basis of their solubility they may be classified into three groups.

1. The acetone-soluble lipoids.
2. The acetone-insoluble, but alcohol-soluble.
3. The acetone-insoluble and alcohol-insoluble.

These differences in solubility are referred not to the pure substances but to material obtained by the extraction of the tissues with ether.

In this laboratory,[4] it was found that the first fraction consisted principally of lecithin with small proportions of cephalin which can be readily removed; the second fraction consisted of lecithin containing larger proportions of cephalin; the third con-

[1] Drechsel, E., *J. prakt. Chem.*, 1886, xxxiii, 425.

[2] Baskoff, A., *Z. physiol. Chem.*, 1908, lvii, 395.

[3] Frank, A., *Biochem. Z.*, 1913, l, 277. Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1916, xxiv, 115.

[4] Levene, P. A., and Komatsu, S., *J. Biol. Chem.*, 1919, xxxix, 91.

sisted of very small proportions of lecithin and larger proportions of cephalin, but the bulk of the material of this fraction is composed of fragments of the other lipoids.

The present investigation aimed to find whether or not the enumerated three fractions with their general characteristics can be obtained from the liver tissue. The answer obtained was in the affirmative.

The acetone-soluble fraction led to the preparation of perfectly pure lecithin. The acetone-insoluble but alcohol-soluble fraction led to a lecithin and cephalin mixture in which cephalin constituted about 20 per cent of the material. From this fraction also practically pure lecithin could be prepared.

The acetone-insoluble and alcohol-insoluble fraction was found to be a mixture of little lecithin, little cephalin, and of a variety of fragments of these and perhaps of other lipoids.

In this respect, then, the lipoid material obtained from the liver does not differ from that of other organs.

The study of the details of the structure of liver lecithin on one hand, and of the cephalin fraction on the other, however, has brought forward some new facts, which are important not only in reference to the special topic of liver lipoids but to that of the unsaturated lipoids in general.

Liver Lecithin.—The point of interest in connection with this substance lies in the nature of the fatty acids entering into the structure of its molecule. In the literature on this subject there exists utter confusion. A critical review of the subject will be given in a future publication. Here it suffices to note that liver lecithin contains in its molecule two fatty acids: one saturated stearic acid and the other unsaturated of the linolic series. There was no evidence of the presence of oleic acid. This conclusion was reached on the basis of the solubility of the barium salt of the unsaturated acid in ether, and on the basis of the hydrogen and iodine values of the fatty acid and of lecithin.

The hydrogen number (grams of hydrogen absorbed by 100 grams of substance) of a lecithin containing in its molecule linolic acid is 0.5018.

The number found for our lecithin was 0.5018.

The iodine number for two double bonds is 63.26.

The value we found was 72.73.

For linolic acid the hydrogen number is 1.439.

Our estimations on the unsaturated fatty acids from lecithin gave an average of 1.508.

The calculated iodine value is 180 and we found 210.

Whether or not the acid is linolic or its higher homologue is not absolutely certain. The analytical data obtained on the hydrogenated acid prepared in the course of the present work correspond to that required by theory for the higher homologue with C_{20}. Before a definite conclusion is reached a larger quantity of material is required, which will permit a more rigorous purification.

In this respect the lecithins obtained from the acetone-insoluble and from the acetone-soluble fractions are identical. The lecithin referred to in this work contained no free amino nitrogen.

Cephalin Fraction.—The point of interest in connection with this fraction is the following. As mentioned before, this fraction represents a mixture of different substances among which lecithin and cephalin are present. The special interest of the present work centers on the cephalin. A sample of cephalin having the elementary composition required by theory for this substance has not been obtained. Levene and West had obtained a sample of hydrogenated cephalin with an elementary composition required by theory. This finding established the correctness of the accepted theory of the structure of cephalin. For biological purposes, however, it still remains of importance to prepare a non-hydrogenated cephalin with a theoretical elementary composition. As yet this object has not been attained, but considerable progress towards this end is reported in this communication. Substances with elementary composition approaching closely the one required by theory and containing between 45 to 70 per cent of cephalin and the remaining part lecithin have been prepared.

On hydrogenation of this material a sample was obtained containing 80 per cent of hydrocephalin. The reduced material on hydrolysis yielded stearic acid and aminoethanol.

In a general way the preparation of this material is as follows: Crude cephalin is dissolved in glacial acetic acid. To this solution 99.5 per cent alcohol is added as long as a precipitate is formed. The filtrate is concentrated under diminished pressure

and the residue is emulsified in water and precipitated by means of acetone. The precipitate is the substance mentioned.

The details of the method are given in the experimental part.

A detailed study of the remaining portion of the cephalin fraction will be presented in a separate publication. The application of this new method of fractionation of the cephalin fraction to material obtained from other organs is in progress in this laboratory.

EXPERIMENTAL.

I. Acetone-Soluble Fraction.

Experiment 1.—400 lbs. of liver were minced, dried, and extracted five times with acetone, followed by an equal number of times with moist ether. The ether extracts were concentrated and precipitated with acetone. The precipitate was dissolved in ether and reprecipitated with acetone, the latter operation being repeated several times. All the acetone-ether solutions thus obtained were united with the original acetone extract, concentrated, allowed to stand at 10°C., and filtered. The filtrate was further concentrated *in vacuo* and poured into a large volume of acetone. On standing at 10°C., a syrupy precipitate formed which was separated by decantation. The acetone solution was again concentrated *in vacuo*. The residue was diluted with a little alcohol and precipitated with an alcoholic solution of cadmium chloride as long as a precipitate formed. After standing over night, the precipitate was filtered, redissolved in ether and water as usual, and reprecipitated with alcohol. The precipitate thus obtained weighed 250 gm. and contained 4.5 per cent of its total nitrogen in the form of amino nitrogen.

200 gm. of the above product were heated with 1.5 liters of toluene, allowed to cool, and centrifuged. A solution, No. 180, and a precipitate, No. 181, were thus obtained. The toluene solution was stirred into 6 liters of ether to which 1 per cent of water had been added. The precipitate, No. 180, was separated and washed with alcohol. The yield was 56 gm. of a substance giving the following analysis.

0.1026 gm. of substance gave on combustion 0.1750 gm. of CO_2, 0.0692 gm. of H_2O, and 0.0240 gm. of ash.

0.1898 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 1.86 cc. of 0.1 N acid.

0.2848 gm. of substance gave on fusion 0.0314 gm. of $Mg_2P_2O_7$.
0.4746 " " " " " " 0.1340 " " $CdSO_4$.

2 gm. of substance were hydrolyzed for a determination of the nitrogen distribution with 10 per cent HCl, neutralized, and then concentrated to 10 cc.

5 cc. of this solution for Kjeldahl determination required 3.95 cc. of 0.1 N acid for neutralization.

2 cc. of this solution gave no nitrogen gas by a Van Slyke determination.

	Calculated for $C_{44}H_{88}O_9NP \cdot 2CdCl_2$	Found.
	per cent	*per cent*
C	45.13	46.51
H	7.35	7.55
N	1.19	1.36
P	2.65	3.06
Cd	19.20	15.17
$\frac{NH_2\ N}{Total\ N}$	$\frac{0}{100}$	$\frac{0}{100}$

The precipitate, No. 181, mentioned above, dissolved for the most part in 500 cc. of benzene. The filtered solution was poured into 95 per cent alcohol and a precipitate, No. 181, was obtained. The yield was 40 gm. of a substance containing 0.5 per cent of its total nitrogen in the form of amino nitrogen.

1.5 gm. of substance were hydrolyzed for a determination of the nitrogen distribution with 10 per cent HCl, neutralized, and then concentrated to 10 cc.

5 cc. of this solution required 3.40 cc. of 0.1 N acid for neutralization in Kjeldahl determination.

2 cc. of this solution in a Van Slyke determination gave 0.02 cc. of N gas, $P = 760$ mm., $T = 23°C$.

Experiment 2.—In a second experiment the precipitation of the concentrated acetone liquors with acetone was omitted. In this case, however, it was more difficult to obtain a fraction free from amino nitrogen. After repeated solution in benzene and precipitation with moist ether, two fractions were obtained. Of these, one consisting of 50 gm., was amino nitrogen-free, and the other, 95 gm., contained 0.5 per cent of its total nitrogen in the form of amino nitrogen.

20 gm. of the lecithin-cadmium chloride compound was freed from cadmium chloride by the method described by Levene and West. The lecithin thus obtained was purified once by Mac-

Lean's water-acetone method. Attempts to reduce the product failed. Hence the purification with water and acetone was repeated four times, after which the precipitate was dissolved in ether and reprecipitated with dry acetone.

The following analytical figures on the unreduced material were obtained.

0.1074 gm. of substance gave on combustion 0.2556 gm. of CO_2, 0.1014 gm. of H_2O, and 0.0102 gm. of ash.

0.1940 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 2.71 cc. of 0.1 N acid.

0.2910 gm. of substance gave on fusion 0.0373 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{44}H_{86}O_9NP$. *per cent*	Found. *per cent*
C	65.75	65.72
H	10.70	10.70
N	1.74	1.97
P	3.86	3.61

0.9733 gm. of substance dissolved in 95 per cent alcohol containing one drop of acetic acid was reduced with hydrogen in the presence of palladium by the method of Paal. The palladium and most of the alcohol was saturated with hydrogen before the introduction of the substance. 59.7 cc. of hydrogen gas at 767 mm. pressure and 21°C. were absorbed (54 cc. were absorbed in 1 hour and 15 minutes, and after 4 hours the figures given above were obtained). Reduced to standard conditions, 59.7 cc. at above temperature and pressure are equivalent to 54.4 cc. or 0.00489 gm. of hydrogen gas.

0.3106 gm. of substance absorbed 0.2259 gm. of iodine when titrated according to the methods of Wijs.

	Calculated for $C_{44}H_{86}O_9NP$.	Found.
Hydrogen number	0.5018	0.5018
Iodine "	63.26	72.73

Fatty Acids of Lecithin.

52 gm. of No. 180 and 38 gm. of No. 181 were combined, powdered, and boiled 8 hours with 700 cc. of 10 per cent hydrochloric acid. After cooling, the cake of fatty acids was filtered off. The acids were repeatedly melted in hot water, allowed to cool, and separated from the water, until the wash water was neutral to litmus. The washed acids weighed about 42 gm. They were converted into barium salts by the method described

by Levene and Meyer,[5] using, however, an aqueous solution of barium hydroxide instead of a methyl alcoholic solution as described in the original method. The barium salts were extracted with ether until practically nothing further was taken up by the solvent. A residue, No. 185, and a solution, No. 186, of barium salts were thus obtained. The barium salts of the saturated acids, No. 185, were decomposed with 10 per cent hydrochloric acid on the water bath, and washed free from inorganic acids. The fatty acids, weighing nearly 21 gm., were converted into lead salts by adding an aqueous solution of lead acetate to a methyl alcoholic solution of the acids, followed by a few drops of ammonia water. The lead salts were filtered and washed with water and acetone. The salts were then dissolved in hot benzene and decomposed with hydrogen sulfide. After removing the lead sulfide, the benzene solution was allowed to stand in an ice chest and a precipitate, No. 185, was obtained. The fatty acids in this fraction melted at 68–68.5°C. (corrected) heated at 5 to 6 seconds per degree. After one recrystallization from benzene, the acid melted at 69–70° (corrected). All melting points recorded in this report were taken at such a rate that 5 to 7 seconds were consumed for each degree rise.

The analysis on No. 185 was as follows:

0.1000 gm. of substance gave on combustion 0.2802 gm. of CO_2 and 0.1170 gm. of H_2O.

1.1546 gm. of substance in a molecular weight determination, dissolved in 10 cc. of toluene and 25 cc. of methyl alcohol, required for neutralization 8.08 cc. of 0.5 N alkali.

	Calculated for $C_{15}H_{30}O_2$. *per cent*	Found. *per cent*
C	75.98	76.41
H	12.76	13.09
Molecular weight	284	285

The combined benzene filtrates from the above acid were concentrated, and a small volume of acetone was added. After standing in an ice chest, the fatty acids were filtered off and dried. The acids in this fraction melted at 60°C. (corrected) and had the following composition.

[5] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1917, xxxi, 627.

0.1005 gm. of substance gave on combustion 0.2809 gm. of CO_2 and 0.1165 gm. of H_2O.

1.2156 gm. of substance in a molecular weight determination titrated as above required for neutralization 8.82 cc. of 0.5 N NaOH.

	Calculated for $C_{18}H_{36}O_2$. *per cent*	Found. *per cent*
C	75.98	76.22
H	12.76	12.97
Molecular weight	284	275

These acids were again converted into lead salts, which were suspended in ether to remove any trace of unsaturated acids. The free acid after liberation was recrystallized from 95 per cent alcohol, and it then melted at 66–67°C. (corrected) and analyzed as follows:

0.1000 gm. of substance gave on combustion 0.2784 gm. of CO_2, 0.1164 gm. of H_2O, and no ash.

0.5831 gm. of substance for a molecular weight determination titrated as above required for neutralization 4.20 cc. of 0.5 N NaOH.

	Calculated for $C_{18}H_{36}O_2$. *per cent*	Found. *per cent*
C	75.98	75.95
H	12.76	13.02
Molecular weight	284	278

This substance was apparently stearic acid containing a small amount of impurities.

The ether solution of the barium salts of the unsaturated acids, No. 186, was decomposed with 10 per cent hydrochloric acid in the presence of benzene and washed free of mineral acid as before. The benzene solution of the fatty acids was filtered, evaporated, and finally the acids were dried in a 90° air bath. 20 gm. of a liquid were thus obtained which remained fluid in the ice chest.

4.9110 gm. of substance, dissolved in 95 per cent alcohol and hydrogenated as previously described, absorbed 1.024 liters of hydrogen at 755.5 mm. pressure and 22°C., or 0.0825 gm. of hydrogen.

0.2386 gm. of substance absorbed 0.5028 gm. of iodine when titrated according to the method of Wijs.

	Calculated for $C_{18}H_{32}O_2$.	Found.
Hydrogen number	1.439	1.679
Iodine "	180	210

100 gm. of the same sample of lecithin cadmium chloride compound from which the free lecithin had been obtained were hydrolyzed as previously described. A sample of the free acids was dissolved in ether and repeatedly washed with water. The ether solution was evaporated and the acids were dried in a steam-heated oven.

0.7961 gm. of the mixed acids was dissolved in 95 per cent alcohol and reduced as previously described. The reduction was nearly completed in 15 minutes. The acids absorbed 75.8 cc. of hydrogen at 762 mm. pressure and 22.6°C., or 0.00611 gm. of hydrogen.

	Calculated for $C_{18}H_{34}O_2$.	Found.
Hydrogen number	0.714	0.768

It was found that the lead salt of oleic acid was readily soluble in ether while the barium salt of this acid was almost insoluble in that solvent. Hence the rest of the fatty acids were converted into lead salts and extracted with ether. The unsaturated acids were liberated in the ether solution with hydrochloric acid and washed free of mineral acid, after which the ether solution was evaporated. The fatty acids were converted into barium salts and extracted with ether. Only a small residue remained after this extraction, and this gave only a trace of fatty acids after decomposition with hydrochloric acid.

The extracted barium salts were decomposed with hydrochloric acid in the presence of ether and washed free of mineral acid. The ether solution was evaporated and dried as previously described. A liquid was thus obtained which on hydrogenation gave the following figures.

0.8114 gm. of substance, dissolved in 95 per cent alcohol and reduced by Paal's method, absorbed 185.2 cc. of hydrogen at 760 mm. pressure and 23°C., or 0.00997 gm. of hydrogen.

	Calculated for $C_{18}H_{32}O_2$.	Found.
Hydrogen number	1.439	1.339

The unsaturated acids were hydrogenated by Paal's method, and the reduced acids purified by converting them into lead salts. Repeated recrystallization from 95 per cent alcohol gave acids which melted at 69–70°C. (corrected). With Kahlbaum's stearic, which had a melting point of 68–69°, it melted at 63°C. The substance had the following composition.

0.1006 gm. of substance gave on combustion 0.2850 gm. of CO_2 and 0.1194 gm. of H_2O.

0.1004 gm. of substance gave on combustion 0.2838 gm. of CO_2 and 0.1184 gm. of H_2O.

1.0718 gm. of substance for a molecular weight determination dissolved in toluene and methyl alcohol required for neutralization 6.72 cc. of 0.5 N NaOH.

	Calculated for $C_{20}H_{40}O_2$. *per cent*	Found. *per cent*
C	76.92	77.25, 77.08
H	12.82	13.28, 13.19
Molecular weight	312	319

Further investigation will be required before a final decision as to the actual composition can be reached.

II. Acetone-Insoluble, Alcohol-Soluble Fraction.

Experiment 3.—A lecithin-cadmium chloride compound almost free from amino nitrogen was obtained from the acetone-insoluble, alcohol-soluble fraction of the lipoids. The solution of the lipoids in alcohol was precipitated with cadmium chloride as previously described, and the precipitate was dissolved in benzene and precipitated with ether saturated with water. The operation was repeated four times. At first a large volume of ether was employed, and it was found necessary to add alcohol to accomplish a satisfactory separation. A yield of 57 gm. of a snow-white substance was thus obtained, which contained 0.42 per cent of its total nitrogen in the form of amino nitrogen.

2 gm. of the substance were hydrolyzed to determine the nitrogen distribution, neutralized, and concentrated to 10 cc.

5 cc. of the solution required for neutralization 4.63 cc. of 0.1 N acid in Kjeldahl determination.

2 cc. of this solution gave 0.02 cc. of N gas in a Van Slyke determination, $P = 755$ mm., $T = 23°C$.

The fatty acids from this fraction were obtained as previously described. The mixed acids had the following characteristics.

1.0025 gm. of substance reduced by Paal's method absorbed 97 cc. of H at 761 mm. pressure and 22.2°C., or 0.00751 gm. of hydrogen.

0.3113 gm. of substance absorbed 0.3217 gm. of iodine when titrated according to the method of Wijs.

	Calculated for $C_{18}H_{34}O_2$.	Found.
Hydrogen number	0.714	0.750
Iodine "	90	103

The saturated acids after one recrystallization from 95 per cent alcohol melted at 6.85° C. (corrected) and had the following composition.

0.1008 gm. of substance gave on combustion 0.2804 gm. of CO_2 and 0.1160 gm. of H_2O.

0.9066 gm. of substance in a molecular weight determination dissolved in toluene and methyl alcohol required for neutralization 6.40 cc. of 0.5 N NaOH.

	Calculated for $C_{18}H_{36}O_2$. *per cent*	Found. *per cent*
C	75.98	75.87
H	12.76	12.88
Molecular weight	284	283

Recrystallized from alcohol, this acid melted at 70-72°C. (corrected). A mixture with Kahlbaum's stearic acid, having a melting point of 68-69°, melted at 68.5°C. (corrected).

The ether solution of the barium salts of the unsaturated acids was evaporated. Attempts to dissolve the residue in pure benzene failed as a gelatinous transparent suspension was formed from which the salts could again be separated by centrifuging. The salts dissolved readily in benzene containing 5 per cent absolute alcohol, from which they were precipitated by pouring the solution into a large volume of absolute alcohol. The substance was dried in vacuum over sulfuric acid, at temperature of boiling water, and had the following barium content.

0.0973 gm. of substance gave on fusion 0.0322 gm. of $BaSO_4$.

	Calculated for $C_{36}H_{62}O_4Ba$. *per cent*	Found. *per cent*
Ba	19.76	19.48

After allowing the barium salts of the above acids to stand in a desiccator over sulfuric acid for more than a week, their solubility in ether had decreased, and the free acids obtained by decomposition with hydrochloric acid formed a viscous syrup. Oxidation

had evidently taken place for on hydrogenation the following figures were obtained.

0.6275 gm. of substance reduced by Paal's method absorbed 71.3 cc. of H at 760 mm. pressure and 22.8°C. or 0.00571 gm. of hydrogen.

0.3455 gm. of substance absorbed 0.4161 gm. of iodine when titrated according to the method of Wijs.

	Calculated for $C_{18}H_{32}O_2$.	Found.
Hydrogen number	1.439	0.911
Iodine "	180	120

Experiment 4.—Another sample of acetone-insoluble, alcohol-soluble lipoid was purified by water and acetone, dried, dissolved in glacial acetic acid, and poured into a large volume of dry acetone. The mixture was allowed to stand at 0°C. over night, when a small precipitate, No. 68, was obtained. This substance had the following composition.

0.1002 gm. of substance gave on combustion 0.2227 gm. of CO_2, 0.0823 gm. of H_2O, and 0.0107 gm. of ash.

0.1960 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 3.14 cc. of 0.1 N acid.

0.2940 gm. of substance gave on fusion 0.0377 gm. of $Mg_2P_2O_7$.

1 gm. was hydrolyzed in estimating the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required 2.92 cc. of 0.1 N acid for neutralization in Kjeldahl determination.

2 cc. of this solution gave on Van Slyke determination 1.48 cc. of N gas; $P = 764$ mm., $T = 21$°C.

Found: C = 61.23, H = 9.44, N = 2.30, P = 3.66 per cent.

$$\frac{NH_2\ N}{\text{Total N}} = \frac{51.4}{100}$$

The acetic acid-alcohol filtrate from No. 68 was evaporated *in vacuo* to a thick syrup, keeping the temperature as low as possible. The residue was emulsified with water and precipitated with a small volume of acetone. The precipitate was dissolved in ether, reprecipitated by acetone, and dried. This substance had the following composition.

0.1059 gm. of substance gave on combustion 0.2523 gm. of CO_2, 0.0971 gm. of H_2O, and 0.0101 gm. of ash.

0.2882 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 4.39 cc. of 0.1 N acid.

0.2885 gm. of substance gave on fusion 0.0405 gm. of $Mg_2P_2O_7$.

0.5 gm. was hydrolyzed for an estimation of the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required 2.16 cc. of 0.1 N acid for neutralization in Kjeldahl determination.

2 cc. of this solution in a Van Slyke determination gave 0.33 cc. of N gas; $P = 768$ mm., $T = 24°C$.

Found: C = 65.38, H = 10.12, N = 2.17, P = 3.92 per cent.

$$\frac{NH_2N}{\text{Total N}} = \frac{13.7}{100}$$

Experiment 5.—Another sample of crude lecithin, which had not been fractionated with acetic acid-acetone, was united with the substance of the above composition and reduced by the method of Paal. After the palladium was removed, the alcoholic solution was allowed to stand in an ice chest when the lipoids crystallized and were filtered off. 56 gm. of lipoids thus obtained were dissolved in chloroform and poured into several volumes of dry ether. The precipitate, weighing 48 gm., was dissolved in 750 cc. of a mixture of methyl ethyl ketone and chloroform as described by Levene and West.[6] 31 gm. separated when the mixture was allowed to stand at room temperature over night. This substance had the following composition.

0.1046 gm. of substance gave on combustion 0.2544 gm. of CO_2, 0.1040 gm. of H_2O, and 0.0104 gm. of ash.

0.2980 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 3.54 cc. of 0.1 N acid.

0.2944 gm. of substance gave on fusion 0.0396 gm. of $Mg_2P_2O_7$.

0.5 gm. was hydrolyzed for determination of the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required for neutralization 2.06 cc. of 0.1 N acid for a Kjeldahl estimation.

2 cc. of this solution gave in a Van Slyke determination 0.52 cc. of N gas; $P = 773$ mm., $T = 25°C$.

Found: C = 67.27, H = 11.28, N = 1.70, P = 3.79 per cent.

$$\frac{NH_2N}{\text{Total N}} = \frac{25.8}{100}$$

The filtrate from the above compound was allowed to stand in the ice chest, when a substance of the following composition separated.

[6] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1918, xxxv, 285.

0.1035 gm. of substance gave on combustion 0.2499 gm. of CO_2, 0.0964 gm. of H_2O, and 0.0094 gm. of ash.

0.1950 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 2.74 cc. of 0.1 N acid.

0.2925 gm. of substance gave on fusion 0.0407 gm. of $Mg_2P_2O_7$.

1 gm. was hydrolyzed for estimation of the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required for neutralization 4.72 cc. of 0.1 N acid for Kjeldahl determination.

2 cc. of this solution used for Van Slyke determination gave 0.90 cc. of N gas; $P = 765$ mm., $T = 22°C$.

Found: C = 66.0, H = 10.46, N = 1.96, P = 3.87 per cent.

$$\frac{NH_2\,N}{\text{Total N}} = \frac{19.3}{100}$$

III. Cephalin Fraction.

The lipoids precipitated by pouring their solution in ether into a large volume of alcohol were extracted four times with 95 per cent alcohol at 60°C. for 40 minutes as described by Levene and Komatsu.[4] The residue was dissolved in ether, precipitated with acetone, and dried. This substance, No. 6, had the following composition.

0.0968 gm. of substance on combustion gave 0.2180 gm. of CO_2, 0.0750 gm. of H_2O, and 0.0122 gm. of ash.

0.1906 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 2.84 cc. of 0.1 N acid.

0.2858 gm. of substance gave on fusion 0.0400 gm. of $Mg_2P_2O_7$.

Found: C = 63.07, H = 8.96, N = 2.17, P = 4.15 per cent.

The substance was further fractionated by dissolving it in ether and pouring the solution into a large volume of alcohol. Finally the material was separated into two fractions by dissolving it in a small volume of ether and allowing it to stand at 0° for 24 hours. An ether solution, No. 26, and a precipitate, No. 28, were thus obtained. Both Nos. 26 and 28 were then separately dissolved in ether and precipitated with a small volume of alcohol, this operation being repeated several times. After this treatment No. 26 analyzed as follows:

0.1044 gm. of substance gave on combustion 0.2278 gm. of CO_2, 0.0830 gm. of H_2O, and 0.0148 gm. of ash.

0.3155 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 3.04 cc. of 0.1 N acid.

0.2895 gm. of substance gave on fusion 0.0400 gm. of $Mg_2P_2O_7$.

1.4 gm. of substance was hydrolyzed to determine the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required for neutralization 8.05 cc. of 0.1 N acid in a Kjeldahl determination.

2 cc. of this solution used for a Van Slyke determination gave 1.69 cc. of N gas, $P = 757$ mm., $T = 23°C$.

Found: C = 62.86, H = 9.39, N = 1.86, P = 4.06 per cent.

$$\frac{NH_2\ N}{\text{Total N}} = \frac{55.2}{100}$$

No. 28 analyzed as follows:

0.2381 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 2.95 cc. of 0.1 N acid.

0.3000 gm. of substance gave on fusion 0.0514 gm. of $Mg_2P_2O_7$.

1 gm. was hydrolyzed to determine the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required for neutralization in a Kjeldahl determination 3.25 cc. of 0.1 N acid.

2 cc. of this solution used for a Van Slyke estimation gave 1.58 cc. of N gas, $P = 768.2$ mm., $T = 25°C$.

Found: N = 1.73, P = 4.77 per cent.

$$\frac{NH_2\ N}{\text{Total N}} = \frac{48.7}{100}$$

A substance analyzing unusually well was obtained from No. 28 by the following procedure. The material was dissolved in three parts of glacial acetic acid with slight warming. The solution was poured into ten parts of alcohol, the precipitate separating on standing in the cold. This was removed by filtration and the filtrate then concentrated *in vacuo*, without heat, to a thick syrup. Attempts to dissolve the residue in ether and reprecipitate it with acetone failed as the lipoid of this fraction was soluble in acetone in the presence of acetic acid. However, the syrup was emulsified with water and precipitated with a small quantity of acetone as described by MacLean for the purification of lecithin. The precipitate was dissolved in ether and reprecipitated with acetone. This substance had the following composition.

0.1036 gm. of substance gave on combustion 0.2372 gm. of CO_2, 0.0838 gm. of H_2O, and 0.0137 gm. of ash.

0.3519 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 3.54 cc. of 0.1 N acid.

0.2916 gm. of substance gave on fusion 0.0357 gm. of $Mg_2P_2O_7$.

0.8 gm. was hydrolyzed to determine the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required for a Kjeldahl determination 1.56 cc. of 0.1 N acid.

2 cc. of this solution in a Van Slyke estimation gave 1.04 cc. of N gas, $P = 759$ mm., $T = 24°C$.

Found: C = 66.00, H = 9.5, N = 1.487, P = 3.60 per cent.

$$\frac{NH_2\ N}{\text{Total N}} = \frac{66.4}{100}$$

It was later demonstrated that the acetone-insoluble material, precipitated from its solution in ether with alcohol at room temperature, was well adapted as a starting material for obtaining a substance of the above composition. The material was simply dissolved in acetic acid and poured into a large volume of dry alcohol. The filtrate was treated as previously described. On samples where the carbon was still low, the process was repeated, thereby increasing the carbon content, while the ratio of the amino nitrogen to total nitrogen remained the same. It was more difficult to obtain this material from crude cephalin which had been purified by the hydrochloric acid method. The analyses of some of these fractions are given in Table I.

Several samples of this material were combined, dissolved in hot absolute alcohol, and placed in an ice chest. The precipitate was separated by filtration. The filtrate was evaporated *in vacuo*, and the residue was dissolved in hot methyl ethyl ketone. On cooling to room temperature a small amount of a white solid separated. This substance, No. 131, had the following composition.

0.1000 gm. of substance gave on combustion 0.2400 gm. of CO_2, 0.0966 gm. of H_2O, and 0.0100 gm. of ash.

0.1894 gm. used for Kjeldahl nitrogen estimation required 3.96 cc. of 0.1 N acid for neutralization.

0.2841 gm. of substance gave on fusion 0.0366 gm. of $Mg_2P_2O_7$.

1 gm. was hydrolyzed to estimate the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required for neutralization in a Kjeldahl determination 3.25 cc. of 0.1 N acid.

2 cc. of this solution gave in a Van Slyke determination 0.65 cc. of N gas, $P = 752$ mm., $T = 25°C$.

Found: C = 66.78, H = 11.03, N = 2.93, P = 3.66 per cent.

$$\frac{NH_2\ N}{\text{Total N}} = \frac{19.7}{100}$$

The filtrate from No. 131 was evaporated *in vacuo*. The residue was taken up in 95 per cent alcohol, slightly acidified with acetic acid, and reduced by Paal's method. After hydrogenation had proceeded for a time a considerable quantity of solids separated which apparently hindered further catalytic action. The tubes were emptied, the solids were separated by filtration in an ice chest, and the hydrogenation was repeated on the filtrate. The

TABLE I.

No.	C	H	N	P	$\frac{NH_2N}{Total\ N}$
	per cent	*per cent*	*per cent*	*per cent*	
82	64.42	9.92	1.91	3.80	$\frac{45.3}{100}$
95	65.76	9.83	1.52	3.37	$\frac{60.1}{100}$
97	66.00	10.43	1.79	3.58	$\frac{44.1}{100}$
104	64.93	10.03	2.28	3.64	
122	64.78	8.97			$\frac{55.9}{100}$
217	66.46	10.10	2.36	3.48	$\frac{52.2}{100}$
Calculated: for $C_{44}H_{86}O_8NP$ (lecithin)	65.60	10.79	1.74	3.86	$\frac{00}{100}$
for $C_{41}H_{80}O_8NP$	66.17	10.57	1.88	4.17	$\frac{100}{100}$

reduced lipoids thus obtained were dissolved in hot absolute alcohol, filtered from palladium, and allowed to cool to room temperature. The resultant precipitate, No. 132, gave the following analysis.

0.1023 gm. of substance gave on combustion 0.2416 gm. of CO_2, 0.0910 gm. of H_2O, and 0.0113 gm. of ash.

0.1947 gm. used for Kjeldahl nitrogen estimation required for neutralization 2.58 cc. of 0.1 N acid.

0.2948 gm. of substance gave on fusion 0.0406 gm. of $Mg_2P_2O_7$.

1 gm. was hydrolyzed to estimate the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required for neutralization in a Kjeldahl determination 3.55 cc. of 0.1 N acid.

1.5 cc. of this solution gave in a Van Slyke determination 1.94 cc. of N gas, $P = 752$ mm., $T = 24°$C.

Found: C = 65.98, H = 10.30, N = 1.89, P = 3.93 per cent.

$$\frac{NH_2\ N}{\text{Total N}} = \frac{71.8}{100}$$

The filtrate from No. 132 was allowed to stand in an ice chest whereupon a precipitate, No. 137, was obtained. This substance gave the following analysis.

0.1029 gm. of substance gave on combustion 0.2480 gm. of CO_2, 0.0942 gm. of H_2O, and 0.0105 gm. of ash.

0.1944 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 2.03 cc. of 0.1 N acid.

0.2916 gm. of substance gave on fusion 0.0408 gm. of $Mg_2P_2O_7$.

1 gm. was hydrolyzed to determine the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required for neutralization in a Kjeldahl determination 3.45 cc. of 0.1 N acid.

2 cc. of this solution gave in a Van Slyke determination 1.79 cc. of N gas, $P = 750$ mm., $T = 25°$.

Found: C = 66.68, H = 10.36, N = 2.02, P = 3.94 per cent.

$$\frac{NH_2\ N}{\text{Total N}} = \frac{51.1}{100}$$

The filtrate from No. 137 was evaporated. The residue was dissolved in ether and precipitated with acetone. The precipitate, No. 139, gave the following analysis.

0.0944 gm. of substance gave on combustion 0.2232 gm. of CO_2, 0.0906 gm. of H_2O, and 0.0094 gm. of ash.

0.1798 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 2.18 cc. of 0.1 N acid.

0.2697 gm. of substance gave on fusion 0.0368 gm. of $Mg_2P_2O_7$.

0.5 gm. was hydrolyzed to determine the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required for neutralization in a Kjeldahl determination 1.61 cc. of 0.1 N acid.

1.75 cc. of this solution gave in a Van Slyke determination 0.62 cc. of N gas, $P = 773$ mm., $T = 24°$.

Found: C = 65.28, H = 10.87, N = 1.72, P = 3.84 per cent.

$$\frac{NH_2\,N}{\text{Total N}} = \frac{44.6}{100}$$

After washing the reduction tubes with hot alcohol a solid remained attached to the walls, which was not easily removed with that solvent. This material was dissolved in a mixture of chloroform and alcohol and filtered. After evaporation of the solvent, the residue was dissolved in hot absolute alcohol, filtered, and allowed to cool to room temperature. The precipitate, No. 142, gave a clear colorless filtrate after hydrolysis with hydrochloric acid, and this solution did not reduce Fehling's solution. No. 142 had the following composition.

0.1040 gm. of substance on combustion gave 0.2472 gm. of CO_2, 0.0970 gm. of H_2O, and 0.0109 gm. of ash.

0.1954 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 2.28 cc. of 0.1 N acid.

0.2938 gm. of substance gave on fusion 0.0402 gm. of $Mg_2P_2O_7$.

0.5 gm. of substance was hydrolyzed to determine the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required for neutralization in a Kjeldahl determination 1.07 cc. of 0.1 N acid.

2 cc. of this solution gave in a Van Slyke determination 0.87 cc. of N gas, P = 754 mm., T = 23°.

Found: C = 66.05, H = 10.62, N = 1.66, P = 3.87 per cent.

$$\frac{NH_2\,N}{\text{Total N}} = \frac{80.7}{100}$$

10 gm. of No. 139 were hydrolyzed and the bases and acids were obtained as previously[7] described. In this case, however, the extraction with alcohol and acetone was not repeated. 0.035 gm. of amino nitrogen was obtained in the acetone extract, and from this 0.33 gm. of a gold salt was obtained which melted at 188°C. and analyzed as follows:

0.1159 gm. of substance gave 0.0570 gm. of Au on ignition to constant weight.

	Calculated for $C_2H_5ON.AuCl_4$.	Found.
	per cent	*per cent*
Au	49.17	49.18

[7] Levene, P. A., and Ingvaldsen, T., *J. Biol. Chem.*, 1920, xliii, 355.

The residue from the acetone extract gave a picrate which melted at 240–241° and had the following nitrogen content.

0.2448 gm. of substance used for a modified Kjeldahl nitrogen estimation required for neutralization 29.3 cc. of 0.1 N acid.

	Calculated for $C_6H_{13}ON\ C_6H_2O_7N_3$. *per cent*	Found *per cent*
N	16.86	16.75

The fatty acids were obtained as previously described. The acids were once recrystallized from acetone, and then from ether. The substance melted at 68°–69°C. (corrected) and had the following composition.

0.1012 gm. of substance gave 0.2810 gm. of CO_2, 0.1146 gm. of H_2O, and no ash.

1.0294 gm. dissolved in benzene and methyl alcohol in a molecular weight determination required for neutralization 35.3 cc. of a 0.1 N NaOH solution.

	Calculated for $C_{18}H_{36}O_2$ *per cent*	Found. *per cent*
C	75.98	75.72
H	12.76	12.67
Molecular weight	284	291

STRUCTURE OF YEAST NUCLEIC ACID.

AMMONIA HYDROLYSIS: ON THE SO CALLED TRINUCLEOTIDE OF THANNHAUSER AND DORFMÜLLER.

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In a previous communication[1] were presented the theoretical arguments which militated against the conception of the structure of yeast nucleic acid formulated by Thannhauser and Dorfmüller.[2]

In order to add weight to the theoretical considerations, it seemed desirable to demonstrate by experiment that the trinucleotide described by these authors was in reality a mixture of mononucleotides.

These authors state: "Levene regards yeast nucleic acid as a tetranucleotide. If this view is correct then on mild hydrolysis one should obtain the four nucleotides." Instead, Thannhauser and Dorfmüller claimed to have isolated uridinphosphoric acid and a trinucleotide.

Hence, if it were shown by experiment that the four mononucleotides were formed under conditions of hydrolysis employed by these authors, then the theory advanced by them would automatically fall. True, the present writer has demonstrated the cleavage of the molecule of yeast nucleic acid into four mononucleotides under conditions of hydrolysis that are much milder than those employed by Thannhauser and Dorfmüller, yet it seemed desirable to prove that following the exact directions of these authors and employing efficient methods of separation one obtains in reality a mixture of mononucleotides and not a trinucleotide.

[1] Levene, P. A., *J. Biol. Chem.*, 1918, xxxiii, 425; 1919, xl, 415.

[2] Thannhauser, J. S., and Dorfmüller, G., *Z. physiol. Chem.*, 1917, c, 121.

The conditions of hydrolysis employed by Thannhauser and Dorfmüller were the following: 50.0 gm. of nucleic acid were taken up in 140.0 cc. of 25 per cent ammonia solution and boiled with reflux condenser for 2 hours. In the experiments herein reported 500.0 gm. of nucleic acid were taken up in 1,500 cc. of 25 per cent ammonia water and heated with reflux condenser for 30 minutes; thus the time of the action of the alkali was reduced to one-quarter of that employed in the original experiment of Thannhauser and Dorfmüller.

From the product of hydrolysis there were isolated in crystalline form the three mononucleotides entering into the structure of the molecule of nucleic acid and guanylic acid thus far obtained only in an amorphous condition.

These results were to be expected on the basis of the experience reported in previous publications, and they further confirm the conclusion regarding the structure of the molecule of yeast nucleic acid expressed by the present writer.

EXPERIMENTAL.

Conditions of hydrolysis were practically those of Thannhauser and Dorfmüller, save for the duration of the experiment. 500.0 gm. of nucleic acid were taken up in 1,500 cc. of 25 per cent ammonia water. The product of hydrolysis was concentrated under diminished pressure and separated into the guanylic and adenylic fractions following the directions of Jones and coworkers.[3]

Each fraction was converted into its brucine salt and these were fractionated by crystallization out of 35 per cent alcohol and out of methyl alcohol. The fractionation of this material did not proceed as smoothly as that of the material described in previous publications. As a guide the analysis of the brucine salts of various fractions was used. But the analytical differences of various fractions were not so characteristic as corresponding fractions of brucine salts, when these were obtained from material hydrolyzed under conditions described in previous papers.

[3] Jones, W., and Richard, A. E., *J. Biol. Chem.*, 1914, xvii, 71. Jones, W., and Germann, H. C., *J. Biol. Chem.*, 1916, xxv, 93.

From the most soluble brucine salts (soluble in methyl alcohol) adenylic acid was obtained. The brucine salts were converted as usual into the lead salts and these into free adenosinphosphoric acid.

The analytical data on the substance are as follows:

0.1002 gm. of the substance gave 0.1190 gm. of CO_2 and 0.0418 gm. of H_2O.

0.2000 gm. of the substance employed for Kjeldahl nitrogen estimation gave 26.93 cc. of 0.1 N acid.

	Calculated for $C_{10}H_{14}N_5PO_7+H_2O$. *per cent*	Found. *per cent*
C	32.86	32.39
H	4.41	4.67
N	19.16	18.85

The optical rotation of the substance was

$$[\alpha]_D^{20} = \frac{-0.84 \times 100}{1 \times 2} = -42.0°$$

The mother liquor for the crystalline adenosinphosphoric acid on concentration gave a crystalline deposit resembling cytosinphosphoric acid. The substance was recrystallized out of a solution of one part of water to three of ethyl alcohol, and analyzed as follows:

0.1096 gm. of the substance gave 0.1360 gm. of CO_2 and 0.467 gm. of H_2O.

0.2000 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 18.87 cc. of 0.1 N acid.

	Calculated for $C_9H_{14}N_3PO_8$. *per cent*	Found. *per cent*
C	33.42	33.85
H	4.37	4.67
N	13.00	12.86

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{+0.87 \times 100}{1 \times 2} = +43.5$$

The uridinphosphoric acid was obtained from the most insoluble fraction of brucine salts. The brucine salt was converted into ammonium salts. The sample, however, analyzed for a mixture of

mono- and di-basic ammonium salt. The mother liquor of the ammonium salt was transformed into a lead salt which, dissolved on boiling and on slow cooling, settled out in crystals of the appearance of the lead salt of uridinphosphoric acid.

The salt analyzed as follows:

0.1000 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 3.96 cc. of 0.1 N acid.

	Calculated for $C_9H_{11}N_2PO_9Pb$. *per cent*	Found. *per cent*
N	5.29	5.54

Guanosinphosphoric acid was obtained only in the gelatinous state, and only because of the small yield the material could not be made to crystallize.

RELATION OF HYDROGEN ION CONCENTRATION TO THE INCOAGULABILITY OF BLOOD PRODUCED BY PEPTONE, HIRUDIN, AND COBRA VENOM.*

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(Received for publication, July 8, 1920.)

The experiments which form the basis of this paper were undertaken at the suggestion of Professor Mathews of this laboratory, with a view to determine, if possible, whether the processes giving rise to an inhibition of the coagulability of the blood through the agencies of peptone, hirudin, and cobra venom are accompanied by any alterations in the hydrogen ion content of the blood, and, if so, whether these changes might throw any light on the processes taking place. The results show that when incoagulability is produced by the intravenous injections of peptone, there occurs a very marked acidity which is absent when the incoagulability is brought about by similar injections of hirudin and cobra venom. Evidences adduced from experiments with the latter two substances indicate that their anticoagulant power is much diminished with an increase of acidity.

INTRODUCTION.

The properties of the three substances in question and their probable mode of action in the organism have been the subject of much experimental work. Many of these investigations have been made on animals of the most diverse types, and have given

* The work reported in this paper was done some time ago at the University of Chicago. The publication was delayed in the hope of adding to and elucidating some of the data. The intervention of the war delayed the publication still further.

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October, 1919.

rise to many contradictory statements and widely differing theories. However, since the results outlined in the following pages have been obtained from a study of dog blood only, and deal with the physicochemical conditions under which the peptone, hirudin, and venom act, or which they create in the blood, rather than the compounds with which they react, a review of the literature pertaining to these substances is not given.

Methods.

All estimations of the reaction of the blood were made by means of the gas chain method, and since the procedure adopted has been that described by Michaelis (1) in detail, no description of it will be given here. The electrode vessels used were the Michaelis U-tubes into which the blood was allowed to flow from cannulas inserted into the vessels of the animal. In the case of the ear veins the blood passed from an incision in the vessels directly into the hydrogen electrode. The readings obtained with these tubes were frequently supplemented by others made with the Hasselbalch electrode which gives slightly more nearly correct values when the fluids examined contain large quantities of CO_2. Very small differences, practically negligible, were found between the values obtained with either electrode. This is probably due to the small percentage of CO_2 which the peptone blood contains, as has been shown by Salviolo (2) and by Lahousse (3). Unless otherwise mentioned, coagulation was considered complete when the tube containing clotted blood could be inverted without loss of contents. This method of estimating coagulation, although less exact than others, was employed because a minimum amount of time was required in manipulation. Throughout the series, dogs were used exclusively, and these were not fed for 24 hours preceding each experiment.

Further description of methods employed is given in connection with the various groups of experiments into which the paper is divided.

RESULTS AND DISCUSSION.

1. Experiments Showing Changes in Alkalinity Following Injection of Peptone.

Previous to each experiment the reaction of normal dog blood was estimated in blood obtained from the vessels of the ear. The dog was then anesthetized with ether and, during the anesthesia, cannulas were inserted into the external jugular vein and the carotid artery. The animal was then allowed to recover for 1 hour in order to avoid any possible alteration in reaction of the blood resulting from the anesthetic which has been shown by Menten and Crile (4) to cause a marked increase in acidity persisting for a period not longer than 45 minutes. On complete recovery, the required amount of 9 per cent Witte's peptone in 0.85 per cent NaCl was run into the jugular vein from a burette and duplicate samples of blood were then drawn immediately and subsequently at varying intervals from the carotid artery. One sample was observed for the coagulation time and on the other measurements of the alkalinity were made. Following the injection of the peptone in quantities greater than 0.1 gm. per kilo of dog, the blood rapidly develops a marked increase in acidity, of which the degree and the rapidity of onset are largely dependent upon the amount of peptone injected. The greater the percentage of peptone per kilo of animal, the more profound is the reaction and the more rapidly does a fatal acidity of the blood develop. This is illustrated by Protocols 1 and 2 which are typical of the five experiments made.

Protocol 1.—Dog weighed 5 kilos. 1 hour after cessation of ether administration 30 cc. of 9 per cent Witte's peptone in 0.85 per cent NaCl (0.54 gm. of peptone per kilo) were injected into the external jugular vein. Blood withdrawn from the carotid artery shows the values given in Table I. Normal blood clotted in 3½ minutes, but all samples of peptone blood were uncoagulated at the end of 48 hours.

Protocol 2.—Dog weighed 4,400 gm. 1 hour and 10 minutes after the cessation of ether administration 95 cc. of 9 per cent Witte's peptone in 0.85 per cent NaCl (2.11 gm. per kilo) were rapidly injected into the external jugular vein. Samples of blood withdrawn from the carotid artery remained uncoagulated in the ice box at the end of a week and gave the reactions recorded in Table II.

TABLE I.

	Blood.	Blood $H^+ \times 10^{-7}$
	pH	
Normal blood from ear vein.....	7.41	0.39
3 min. after peptone injection.....	7.38	0.42
6 " " " "	7.33	0.47
8 " " " "	7.31	0.49
11 " " " "	7.19	0.65
15 " " " "	7.16	0.70
21 " " " "	7.14	0.73
26 " " " "	7.11	0.79
29 " " " "	7.09	0.82

TABLE II.

	Blood.	Blood $H^+ \times 10^{-7}$
	pH	
Normal blood from ear vessels.....	7.31	0.49
5 min. following peptone injection.....	7.09	0.82
7 " " " "	7.02	0.96
15 " " " "	6.94	1.16
17 " " " " Dog died.		

When a smaller amount of peptone was injected, the maximum acidity did not reach such a high value, and the blood in a short time regained its normal reaction, as is shown in Protocol 3.

Protocol 3.—Dog weighed 8,700 gm. Into a cannula in the external jugular vein 40 cc. of 8 per cent Witte's peptone in 0.85 per cent NaCl (0.37 gm. per kilo) were rapidly run from a burette. Blood from the carotid artery gave the reactions recorded in Table III.

TABLE III.

	Coagulation time.	Blood.	Blood $H^+ \times 10^{-7}$
		pH	
Normal blood from ear.....	3 min., 40 sec.	7.43	0.37
6 min. after injection of peptone.	Between 3 and 4 hrs.	7.46	0.35
9 " " " " "	" 3 " 4 "	7.31	0.99
11 " " " " "	" 4 " 5 "	7.27	0.54
14 " " " " "	" 4 " 5 "	7.13	0.74
17 " " " " "	" 4 " 5 "	7.15	0.70
19 " " " " "	" 4 " 5 "	7.18	0.66
21 " " " " "	15 min.	7.37	0.34

With doses of peptone less than 0.5 gm. per kilo of animal immediately following injection there is produced a suspension of coagulability parallel with which an increased acidity is developed in the blood. The period of time during which the coagulant power of the blood is suspended varies between 15 and 20 minutes, an observation already reported by Gley and Pachon (5) and other workers. At the end of this period there is a return to the normal blood reaction, with a loss of the acquired property of incoagulability.

TABLE IV.

	Coagulation time.	Blood	Blood $H^+ \times 10^{-7}$
		pH	
Normal blood from ear.....	3½ min.	7.44	0.37
" " " " during artificial respiration.....	3½ "	7.46	0.35
Blood taken 4 min. after peptone injection.	Not coagulated in 5 hrs.	7.51	0.31
7 " " " "	" " " 5 "	7.34	0.46
10 " " " "	" " " 5 "	7.23	0.59
13 " " " "	" " " 5 "	7.22	0.61
18 " " " "	" " " 5 "	7.34	0.46
23 " " " "	32 min.	7.34	0.46
30 " " " "	5 "	7.38	0.42

In order to ascertain whether these changes were not primarily due to changes in respiration, experiments were performed in which, following severing of the spinal cord, artificial respiration was instituted so there could be no lack of respiratory exchange in the lung due to decreased oxygen tension in the alveoli. That the acidity is not due to this cause is clearly indicated by Protocol 4. Three similar experiments gave analogous results.

Protocol 4.—Dog weighing 8 kilos was anesthetized with ether and cannulas were inserted into the external jugular vein and carotid artery. The spinal cord was severed and artificial respiration commenced immediately. 10 minutes subsequently 31 cc. of 9 per cent Witte's peptone in 0.85 per cent NaCl (0.35 gm. of peptone per kilo) were injected into the external jugular. The blood gave the reactions recorded in Table IV.

Contejean (6) who in his experiments, previous to the injections of peptone severed the spinal cord for the purpose of exclud-

ing the possible influence of the nervous system on the retardation of coagulation caused by peptone, also reports no appreciable difference in the incoagulability obtained when this procedure was employed.

In order to ascertain whether there was developed in the plasma a rise in the acidity coincident with that occurring in the blood, a number of experiments were performed in which readings were made on both fluids. In the five experiments made the changes in the reaction of the plasma were also found to be marked as is shown in Protocol 5.

TABLE V.

	Blood.	Blood $H^+ \times 10^{-7}$.	Plasma.	Plasma $H^+ \times 10^{-7}$
	pH		*pH*	
Normal blood from ear vessel.....	7.48	0.33	8.13	0.07
2 min. following peptone injection....	7.43	0.37	8.03	0.09
4 " " " "	7.40	0.40	7.96	0.11
8 " " " "	7.37	0.43	7.92	0.12
12 " " " "	7.32	0.48	7.89	0.13
15 " " " "	7.28	0.53	7.87	0.14
19 " " " " ...	7.22	0.61	7.84	0.15
23 " " " "	7.15	0.71	7.79	0.16
28 " " " "	7.09	0.82	7.75	0.18
35 " " " "	7.16	0.70	7.72	0.24

Protocol 5.—3 hours after the cessation of ether administration, there was injected into the external jugular of a dog weighing 8,500 gm. 40.7 cc. of Witte's peptone in 0.85 per cent NaCl (0.42 gm. per kilo). Measurements of the blood and plasma gave values which are typical of the five cases (Table V).

24 hours later, none of these samples was coagulated except the last in which small shreds of clot were beginning to form about the surface of the test-tube.

The experiments made with Witte's peptone demonstrate that coincident with the retardation or inhibition of coagulation, there is a marked increase in the acidity of the blood and of the plasma, and that this increase is proportional to the amount of peptone injected and the degree of incoagulability produced. These results on the change in acidity are in harmony with those reported by Salviolo (2) and by Lahousse (3), both of whom

found by titration an increased acidity with a decreased CO_2 tension in the blood. Their observations were confirmed by the later publications of Athanasin and Carvallo (7) on this subject and are in direct opposition to those reported by Dastre and Floresco (8).

II. Experiments on Changes in Blood Reaction Caused by Injections of Peptone Subsequent to the Removal of the Liver.

That the liver plays an important rôle in the production of incoagulability with peptone has been shown by numerous observers. Contejean (9), Gley (10), Gley and Pachon (11), Delezenne (12), and Nolf (13) have demonstrated that removal of this organ from the circulation previous to injection of peptone solution prevents the development of incoagulability of the blood. With a view to determining whether the increased acidity which peptone develops with the liver intact could be duplicated in the animal from which the liver was removed, a number of experiments were performed. It is very difficult to remove the entire liver "en bloc" in a dog without occluding the vena cava. On this account each lobe was tied off separately as close as possible to the hilum of the liver with a wide tape. With even a considerable amount of the liver *in situ* with such doses as 0.35 gm. per kilo of animal, incoagulability does not occur and very little change in the blood is demonstrable; with doses as high as 0.7 gm. per kilo of animal a transient incoagulability may be produced with a simultaneous slight increase in acidity, which persists for a few minutes only. Injections were made in eleven animals from which the livers had been removed, and the results were as indicated in Protocol 6.

Protocol 6.—Dog weighed 7 kilos. During ether anesthesia, the lobes of the liver were tied off and cannulas were inserted in the external jugular vein and carotid artery. After the animal was allowed to recover from the anesthetic for 1¼ hours, 42 cc. of 9 per cent Witte's peptone in 0.85 per cent NaCl (0.54 gm. per kilo) were injected into the jugular vein (Table VI).

The specimens in Table VI were kept fluid by the addition of hirudin which Michaelis (1) and subsequently others have shown to have no influence on the reaction of the whole blood.

The effect which is produced when small amounts of liver substance have been left in the animal after ligation of the lobes is illustrated by the values of the blood reaction obtained in a dog weighing 6,300 gm. which was given in a similar manner 0.60 gm. per kilo of a 9 per cent peptone solution in normal saline solution, 1½ hours after removal of the liver (Table VII).

TABLE VI.

	Coagulation time.	Blood.	Blood $H^+ \times 10^{-7}$
	min.	*pH*	
Normal blood from ear vein	4	7.42	0.37
" " " " " after removal of liver	4	7.41	0.39
6 min. after peptone injection	5	7.38	0.42
8 " " " "	5	7.37	0.43
12 " " " "	6		
15 " " " "	6	7.32	0.48
19 " " " "	5	7.30	0.50
25 " " " "	5	7.35	0.45
30 " " " "	5	7.35	0.45

TABLE VII.

	Blood.	Blood $H^+ \times 10^{-7}$
	pH	
Normal blood after removal of liver	7.48	0.33
5 min. after peptone injection	7.36	0.44
10 " " " "	7.25	0.57
13 " " " "	7.25	0.57
16 " " " "	7.25	0.57
20 " " " "	7.24	0.53
30 " " " "	7.25	0.57

All the above samples of peptone blood began to clot in 40 min.

In animals in which the liver had been removed for a period of more than 2 hours, an increased acidity developed without a corresponding retardation of coagulation. Protocol 7 is typical of this condition.

Protocol 7.—After the liver had been removed for 2 hours and 55 minutes from a dog weighing 8 kilos, 50 cc. of 9 per cent peptone in normal saline solution (0.56 gm. per kilo) were injected into the jugular vein. Values of blood reactions are recorded in Table VIII.

It is possible that this phenomenon is to be explained by the fact, first suggested by Contejean (6), that, while the greater part of the substance with which the peptone forms a direct combination is elaborated by the liver, other organs also produce this substance to a lesser degree. When the period of time following the extirpation of the liver is prolonged, an accumulation of this material can take place in these organs, or in the circulating fluid, in quantities sufficient to give on peptone injection a reaction approximating that obtained when the liver is intact.

TABLE VIII.

	Coagulation time.	Blood.	Blood $H^+ \times 10^{-7}$
	min.	*pH*	
Normal blood	3	7.40	0.40
" " after removal of liver	3	7.43	0.37
5 min. after peptone injection	10	7.14	0.73
10 " " " "	10	7.15	0.71
15 " " " "	12	7.13	0.75
19 " " " "	12	7.12	0.77
21 " " " "	12	7.12	0.77
26 " " " "	12	7.10	0.81
35 " " " "	13	7.07	0.86

III. Experiments on Changes in the Reaction of Peptone Subsequent to its Perfusion through the Liver.

As has already been mentioned, the fact that peptone owes its anticoagulant property primarily to the influence of the liver has been established by many investigators. By the interaction of two substances, one contained in the peptone and the other occurring in the liver, there is formed apparently a third compound which is the effective agent in the immediate production of incoagulability. None of these three compounds has yet been isolated, but their physiological properties have to some extent been studied, and especially that of the third in its relation to the prevention of coagulation extravascularly. The study undertaken by the writer was to investigate whether the interaction which takes place in the liver between the two substances mentioned would suffice to explain the reduction of alkalinity which accompanies the loss of coagulation, or whether the second

phase which is more directly concerned with the coagulation process itself was responsible for the change in the blood reaction. The first step was to obtain by means of the peptone perfusion through the liver a fluid which, when added to shed blood, retarded or prevented coagulation. The hydrogen ion concentration of the fluid was then measured. The perfusion experiments were performed on the liver *in situ*. For this purpose the animal was anesthetized and all vessels connected with the liver except the hepatic artery (or portal vein) and the descending vena cava below the liver were ligated. The fluid entering at either the portal vein or hepatic artery was passed through the liver and received again from the inferior vena cava into the perfusion apparatus through which it was kept circulating by means of a system of valves regulated by a motor. The whole apparatus was kept at a temperature of 38°C. by means of a water bath. Although attempts were made to maintain by means of an electric pad a normal body temperature in the animal, it rapidly lost heat. Solutions of 9 per cent Witte's peptone in 0.85 per cent NaCl with and without previously washing out the liver with Tyrode's, or normal saline, solution to render the organ blood-free were used through a series of experiments. Oxygenation of the peptone was secured by passing a continuous stream of oxygen through the fluid in the apparatus.[1]

When the quantity of fluid perfused was large it was not possible to demonstrate that the perfusion fluid had acquired any anticoagulant properties during its passage through the liver. When added in concentrations as great as five parts of fluid to one of blood, there was no appreciable retardation of clotting. Further, no uniform results concerning changes in alkalinity were obtained in the five experiments made although the time during which the peptone was perfused varied from a few minutes to an hour.

Since the continuous perfusion of large amounts of peptone solutions (from 500 to 2,000 cc.) through the liver was unsuccessful in giving a fluid capable of diminishing the coagulation time, the expedient of passing small quantities of fluid separately through the liver and testing each for its anticoagulant power

[1] For the perfusion apparatus used I am indebted to Mr. Siegfried Maurer, who also very kindly helped me with the perfusion experiments.

was tried. The results of the five experiments performed are illustrated in Protocols 8 and 9.

Protocol 8.—In a dog weighing 5 kilos, the vessels of the liver, except the portal vein and the inferior vena cava below the liver, were tied. In each of these a cannula was placed and the liver washed out with 100 cc. of 0.85 per cent NaCl. This fluid possessed no anticoagulant property. 100 cc. of peptone solution were then perfused and, after passing through the liver, ten drops of this fluid added to 10 cc. of blood prevented coagulation for 5 hours. The value 0.168, 10^{-6}, which represented the acidity of the peptone solution was reduced to 0.21, 10^{-6}, after perfusion. A second 100 cc. of the same peptone solution possessed no anticoagulant action after its passage through the liver and gave a reaction of 0.42, 10^{-6}, at the end of the perfusion.

TABLE IX.

Time after perfusion commenced.	Amount of peptone fraction.	Ration blood: peptone used for testing coagulation time.	Coagulation time.	Peptone.	Peptone $H^+ \times 10^{-7}$
min.	*cc.*			*pH*	
2	20	1:1, 1:2, 1:3	Clots immediately.	6.73	1.90
4	20	1:1, 1:2, 1:3	Clots immediately.	6.73	1.90
6	10	1:1	1 hr.	6.70	2.02
16	10	1:1	1 "	6.65	2.26
26	10	1:1	10 min.	6.70	2.02
60	10	1:1	Clots immediately.	6.44	3.65
	(Expressed from liver.)	Peptone before perfusion.		6.80	1.60

Protocol 9.—Dog weighed 8 kilos. All the vessels supplying the liver, except the portal vein and inferior vena cava below the liver, were tied off. The 150 cc. of Tyrode's solution used to wash the blood out of the liver had no anticoagulant properties after its exit from that organ. 200 cc. of peptone were then injected into the portal vein, and after passing through the liver various fractions gave the reactions recorded in Table IX.

That the increased acidity coincident with the production of peptone incoagulability is not occasioned by that part of the process taking place in the liver is apparent from the figures in Table IX. One is, therefore, forced to conclude that this feature

of the phenomenon is intimately associated with those factors immediately concerned in coagulation. Control peptone perfusion experiments on kidney and spleen did not in any case result in the production of fluids possessing anticoagulant properties.

IV. Experiments on the Relation of the Hydrogen Ion Content and Hirudin in the Suspension of Coagulation.

The fact that the addition of hirudin to freshly shed blood in quantities sufficient to delay coagulation causes no appreciable change in the hydrogen ion content was first noted by Michaelis (1). Indeed, hirudin may be added in very considerable quantities to blood without any apparent influence on its reaction. The question, therefore, arose as to whether injection of this substance into the circulation would produce alterations in the

TABLE X.

	Blood.	Blood $H^+ \times 10^{-7}$
	pH	
Normal blood from ear vein	7.50	0.32
3 min. after injection of hirudin	7.41	0.39
6 " " " " "	7.45	0.36
9 " " " " "	7.45	0.36
14 " " " " "	7.46	0.35
19 " " " " "	7.43	0.37
24 " " " " "	7.45	0.36
29 " " " " "	7.43	0.37
33 " " " " "	7.45	0.36

alkalinity of the blood similar in character to those obtained when peptone is injected, or would simulate those changes produced on inhibition of coagulation with hirudin *in vitro*.

Five experiments performed gave uniform results of which Protocol 10 is typical.

Protocol 10.—During ether anesthesia cannulas were placed in the external jugular vein and carotid artery of a dog weighing 5 kilos. 1 hour after the cessation of the ether administration, 200 mg. of hirudin in 50 cc. of 0.85 per cent NaCl were run into the jugular vein from a burette. Blood withdrawn from the carotid artery gave the reactions shown in Table X. Duplicate samples of blood remained unclotted at the end of 10 hours.

TABLE XI.

TABLE XI.

m/15 Na_2HPO_4	m/15 KH_2PO_4	H^+ of solution.	Phosphate solution used.	Blood used.	Coagulation time. I (0.0005 gm. hirudin).	II (0.0005 gm. hirudin).	III (0.001 gm. hirudin).	IV (0.0015 gm. hirudin).	V (0.002 gm. hirudin).	VI (0.005 gm. hirudin).	VII (no hirudin).
cc.	*cc.*		*cc.*	*cc.*							
0.0	10.0	$0.32, 10^{-4}$	5	5	20 min.	15 min.	2 min.	Immediately.	22 min.	Immediately.	16 min.*
0.1	9.9	$1.14, 10^{-5}$	5	5	27 "	21 "	4 "	1 min.	22 "	2 min.	15.5 " *
0.25	9.75	$0.51, 10^{-5}$	5	5	36 "	34 "	5 "	3 "	2 "	3 "	15.5 " *
0.5	9.5	$2.58, 10^{-6}$	5	5	40 "	38 "	8 "	5 "	2 "	4 "	15 " *
1.0	9.0	$1.12, 10^{-6}$	5	5	44 "	39 "	10 "	8 "	$2\frac{1}{2}$ "	4 "	15 " *
2.0	8.0	$0.58, 10^{-6}$	5	5	46 "	42 "	14 "	11 "	3 "	4 "	12 "
3.0	7.0	$0.34, 10^{-6}$	5	5	50 "	45 "	15 "	15 "	3 "	6 "	13 "
4.0	6.0	$0.23, 10^{-6}$	5	5	53 "	50 "	17 "	15 "	4 "	7 "	10 "
5.0	5.0	$1.54, 10^{-7}$	5	5	57 "	54 "	20 "	18 "	6 "	9 "	11 "
6.0	4.0	$1.05, 10^{-7}$	5	5	1 hr.	59 "	27 "	22 "	9 "	9 "	5.5 "
7.0	3.0	$0.68, 10^{-7}$	5	5	1 " 6 min.	1 hr.	36 "	26 "	13 "	14 "	4.5 "
8.0	2.0	$0.42, 10^{-7}$	5	5	1 " 22 "	1 " 10 min.	40 "	35 "	15 "	20 "	3.5 "
9.0	1.0	$1.85, 10^{-8}$	5	5	1 " 50 "	1 " 35 "	51 "	40 "	31 "	35 "	3.5 "
9.5	0.5	$0.91, 10^{-8}$	5	5	2 " 30 "	2 " 10 "	1 hr.	59 "	41 "	49 "	3.5 "
9.9	0.1	$0.21, 10^{-8}$	5	5	3 " 20 "	3 "	1 " 15 min.	1 hr. 20 min.	56 "	1 hr.	3.5 "
10.0	0.0	$6.58, 10^{-9}$	5	5	4 " 10 "	3 " 50 "	1 " 48 "	1 " 50 "	1 hr. 21 min.	1 " 30 min.	3.5 "
Coagulation time of untreated blood.....					3 min.	$3\frac{1}{2}$ min.	3 min.	4 min	3 min.	3 min.	$3\frac{1}{2}$ min.

* Clot does not become firm.

TABLE XII.

0.1 N sodium acetate.	0.1 N acetic acid.	Distilled water.	H^+ of the acetate solution.	Acetate solution used.	Blood used.	Coagulation time. I (0.0005 gm hirudin).	II (0.0005 gm. hirudin).	III (0.001 gm. hirudin).	IV (0.0015 gm. hirudin).	V (0.0015 gm. hirudin).	VI (0.0015 gm. hirudin).	VII (0.002 gm. hirudin).	VIII (0.005 gm. hirudin).	IX (no hirudin).
cc.	*cc.*	*cc.*		*cc.*	*cc.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
1	0.1	8.90	$2, 10^{-7}$	5	5	Coagulated within 3 min.	Coagulated in 3 min.	7	12	47	42	14	Uncoagulated. Hemolysis.	5.5
1	0.2	8.80	$4, 10^{-7}$	5	5	" "	" "	6	9	37	27	12	"	5.5
1	0.4	8.60	$8, 10^{-7}$	5	5	Immediately.	Immediately.	5	7	30	21	9	20	5.25
1	0.8	8.20	$1.7, 10^{-6}$	5	5	"	"	5	5	27	18	7	14	5.25
1	1.6	7.40	$3.7, 10^{-6}$	5	5	"	"	3	4	16	17	6	11	5.0
1	3.2 N acetic acid.	5.80	$6.8, 10^{-6}$	5	5	"	"	3	3	15	15	5	8	4.5
1	0.64	8.36	$1.3, 10^{-5}$	5	5	"	"	3	2	14	14.5	4	6	4.5
1	1.28	7.72	$2.6, 10^{-5}$	5	5	"	"	3	2	12	13	4	5	4.0
1	2.56	6.44	$5.2, 10^{-5}$	5	5	"	"	3	2	10	9.5	3	4	3.5
1	5.12	4.88	$1, 10^{-4}$	5	5	"	"	3	2	9.5	9	2	2	3.0
Coagulation time of untreated blood..						3 min.	3 min.	3 min. 20 sec.	4	3	3	3 min., 20 sec.	4	3.0

Measurements recorded in Table X show quite definitely that there is little increase in the acidity corresponding to the incoagulability which develops. Further, the small divergence of value of the reaction of the blood rendered incoagulable by hirudin from that of normal blood indicates the identity of the *in vivo* and *in vitro* action of this substance in the retardation of coagulation.

Since inhibition of coagulation with hirudin may occur with little change in the reaction of the blood, it may be questioned whether changes in the ionic concentration are a factor of any great significance in modifying the process. To test the possible influence of the hydrogen and hydroxy ion concentration on the anticoagulant action of hirudin equal amounts of Sörensen phosphate and acetate mixtures of known acidity and containing definite quantities of hirudin were added to shed blood, and variations in the coagulation time noted. The results of these experiments are given in Tables XI and XII.

The amount of hirudin specified at the beginning of each column denoting coagulation time represents the quantity of hirudin contained in the 10 cc. of blood mixture.

The marked influence of increased alkalinity in enhancing, and increased acidity in lessening, the action of hirudin in delaying coagulation is revealed by the values recorded in these two tables. Further, that other ions besides the hydrogen and hydroxy are of considerable importance in modifying the reaction is evidenced by a comparison of the coagulation time obtained when the acetate and phosphate mixtures used are of the same hydrogen ion concentration.

V. Experiments on Retardation of Coagulation by Cobra Venom.[2]

The first two of these experiments, outlined below, gave negative results because a quantity of venom insufficient to retard coagulation appreciably was used. In the first of these, a dog weighing 8,800 gm. received 2 cc. intravenously and 5 cc. subcutaneously of a 0.1 per cent solution in 0.85 per cent NaCl.

[2] For the cobra venom used in the five experiments made, I am indebted to Prof. Preston Keyes of this Laboratory, who most kindly supplied me with the material.

The animal survived the injection 1½ hours, but at no time was there any change in the coagulation time of the blood which also remained normal in reaction until a few minutes previous to death when the acidity became markedly increased. Similar results were obtained in a second dog weighing 4,400 gm. that received an injection of 2 cc. of 0.1 per cent venom in 0.85 per cent NaCl in the saphenous vein, and lived for 2 hours.

In the three experiments giving positive results, 10 cc. of 0.1 per cent venom in normal saline solution were injected. Each of these animals weighed between 5 and 6 kilos.

TABLE XIII.

	Coagulation time.	Blood.	Blood $H^+ \times 10^{-7}$.
		pH	
Blood withdrawn before anesthesia commenced	3 min., 20 sec.	7.40	0.40
3 min. following venom injection.	Not coagulated at end of 36 hrs.	7.41	0.39
4 " " " "	Not coagulated at end of 36 hrs.	7.38	0.42
6 " " " "	22 hrs.	7.35	0.45
8 " " " "	18 "	7.30	0.50
10 " " " "	Between 5 and 18 hrs.	7.25	0.57
12 " " " "	" 5 " 18 "	7.25	0.57
14 " " " "	5 hrs. 25 min.	7.22	0.61
16 " " " "	2 " 10 "	7.16	0.70
18 " " " "	1 hr. 8 "	7.08	0.84
20 " " " "	5 "	7.01	0.98
22 " " " "	Immediately.	6.93	1.18
23 " " " "			
Dog dying.			

As essentially the same results were obtained with the three animals, only one protocol is reported in detail.

Protocol 11.—Cannulas were inserted in the external jugular vein and the carotid artery of a fox terrier, weighing 3,500 gm., under ether anesthesia. The animal was allowed to recover from the anesthetic for 1 hour when 10 cc. of 0.1 per cent cobra venom were run into the jugular vein from a burette. The coagulation time and reaction of the blood drawn from the carotid immediately after and at varying intervals subsequent to the injection were as shown in Table XIII.

The blood withdrawn at 18, 20, 22, and 23 minutes after the venom injection had a very dark appearance, and this was especially marked in the blood sample with the most acid reaction.

The inimical influence of increased acidity of the blood on the inhibition of clotting by the use of cobra venom intravascularly seems to be established by the above results. As the amount of cobra venom necessary to inhibit extravascular clotting is comparatively large, a limited supply of the venom precluded any experiments on the relation between acidity and incoagulability developing *in vivo*.

VI. CONCLUSION.

From the work outlined in the preceding pages it is evident that the rôles which peptone on the one hand and hirudin and cobra venom on the other play in the prevention of retardation of clotting are of a very different character. With the production of incoagulability by peptone there occurs a marked increase in the acidity of the blood, a condition under which the other two substances lose completely or in large degree their anticoagulant power.

SUMMARY.

1. Coincident with the incoagulability of the blood produced by intravenous injections of Witte's peptone, there is a marked increase in the acidity of the blood and of the plasma.

2. When Witte's peptone is injected into the circulation subsequent to extirpation of the liver, extravascular clotting is not retarded, and the blood shows little change in acidity.

3. Witte's peptone which has acquired anticoagulant properties after perfusion through the liver shows only a slight increase in acidity.

4. The retardation of coagulation resulting from intravenous injection of hirudin is accompanied by little or no change in the acidity of the blood.

5. Increase of acidity of the blood lessens and increase of alkalinity enhances the property of hirudin in delaying clotting *in vitro*.

6. The reaction of the blood is little altered by intravenous injections of cobra venom in quantities sufficient to prevent clotting. With an increase of acidity of the blood its acquired incoagulability is lost.

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GAS PRODUCTION DURING THE ACETONE AND BUTYL ALCOHOL FERMENTATION OF STARCH.

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In continuing an investigation of the production of acetone and butyl alcohol from starch, a detailed study has been made of the changes in the composition of the gas produced during the fermentation period. An attempt has also been made to coordinate the results from this and an earlier portion of the research (1), and to make a contribution to our knowledge of the biochemical mechanism of the fermentation as a whole.

Various observations regarding the composition of the gas formed during the production of butyl alcohol, alone or with acetone, have been recorded in the literature. Beijerinck (2) in his exhaustive study of *Bacillus butylicus* (*Granulobacter butylicum*) observed that H_2 and CO_2 are given off during the fermentation. Near the commencement the mean relative volumes of the gases were about 1 of CO_2 to 4 of H_2. During the time of rapid cell division and butyl alcohol production the relation of the gases was 1 of CO_2 to 1 of H_2. In some cases the percentage volume of H_2 in the gas was further reduced. In the so called "after-fermentation" the percentage volume of H_2 in the gas was found to increase. The organism investigated by Perdrix (3), and named by him "*Bacille amylozyme*," was found to produce a diminishing amount of H_2 as the fermentation proceeded. It is of interest to observe that this organism also produces acetic acid and x butyric acid, the former in larger amounts at the beginning of the fermentation.

In his address on the fermentation of starch by the Weizmann organism Gill (4) stated that: "During the fermentation a mixture of approximately equal volumes of H_2 and CO_2 is evolved," and also, that: "When the fermentation begins, gas consisting of

approximately equal volumes of CO_2 and H_2 is evolved." In their observations regarding the same fermentation Reilly and his collaborators (5) state that during the first half of the fermentation period the composition of the gas evolved changes, owing to a gradual increase in the CO_2 percentage. During the second half the composition remains constant and they attribute the earlier changes to the greater solubility of CO_2 in the medium.

Method.

The rates of various biochemical reactions of the fermentation have previously been observed and correlated, and in order to coordinate the changes in the composition of the gas with these reactions it is necessary to study the changes in relation to one of them. The rate of total gas production was arbitrarily chosen. It was then possible to correlate the observations with the formation of the acid and neutral products of the fermentation.

The rate of gas production was measured by means of the apparatus shown in Fig. 1. This can be assembled from the ordinary equipment of the laboratory and might be of value for class purposes owing to its cheapness and adaptability. The gas given off passes into the large test-tube through *A* and out again through *B*. The latter tube is drawn out to a capillary and in this way the flow of gas is restricted. The slight pressure created in the fermentation vessel is measured by means of the manometer in terms of a column of water. A trial fermentation is necessary in order to adjust the instrument. At the point of maximum gas production the capillary tip is carefully filed until a fairly large pressure is recorded on the manometer; *e.g.*, 8 inches. The rate of gas flow is proportional to the square root of the pressure, and comparative measurements are obtained which are sufficiently accurate for the purpose. If quantitative figures are required the instrument can be calibrated by placing it in series with a reliable experimental gas meter and a supply of gas.

Samples of gas were collected at regular intervals over mercury in the apparatus described by Harden, Thompson, and Young (6). During the early hours of the fermentation the O_2, from the air still present in the vessel, was absorbed by phosphorus. Throughout the fermentation the CO_2 was absorbed by fresh KOH solution, and the H_2 was measured by difference.

The majority of the data collected was obtained from fermentations of 20 liters of mash containing 5 per cent maize meal.

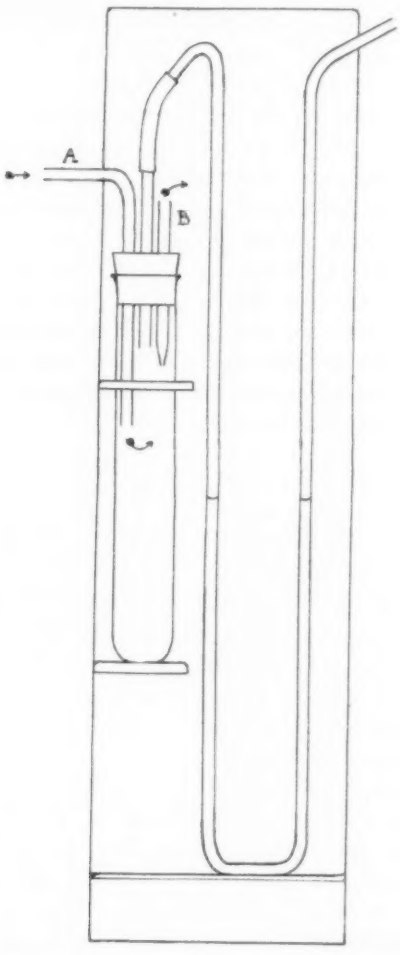

FIG. 1. The actual size of the test-tube is 9 by 1½ inches, and the apparatus as a whole is drawn to scale.

They were conducted in suitably constructed copper vessels which were sterilized in a large autoclave. During the fermen-

tation the vessel was placed in an incubator at 36°C., and a short tube passing through the door connected with the gas apparatus.

EXPERIMENTAL.

Series I.

Each experiment in the series was essentially the same and differed only in that a particular section of the fermentation period was under close observation. By standardizing the conditions as far as possible, a series of fermentations was obtained which differed only slightly in rapidity and total gas production. The results obtained are summarized in Table I, and are represented by curves in Fig. 2. The letters given to the periods of Table I and to the curves in Fig. 2 correspond. The various groups of figures for different periods overlap considerably, and from them it has been possible to construct a typical curve for the entire fermentation period.

Series II.

In connection with the observations on the early hours of the fermentation it was thought desirable, in view of their theoretical significance and the conclusions of other investigators regarding them, to discover if possible whether the gas actually produced during this period was in reality so rich in H_2. There appeared to be a slight possibility that, owing to the gradual saturation of the medium with CO_2, the gas evolved was not representative of the gas being produced.

A smaller volume of mash, 400 cc., was inoculated and allowed to ferment for 5 hours. The gas given off was collected over mercury. The gas in solution at the end of this period was expelled by heat and also collected. The gas produced during the 5 hours was found to contain 74 per cent of H_2. Owing to the larger percentage of culture added to the mash this fermentation was more rapid than those of Series I.

It is considered justifiable therefore to conclude that, as the figures and curves suggest, the gas produced at the immediate commencement of the fermentation is pure H_2. The percentage in the gas immediately begins to fall, due to the production with

TABLE I.

Period.	Date.	Time after inoculation.	Pressure.	$\sqrt{P}$	H_2 in gas evolved.
	1920	*hrs.*	*in.*		*per cent*
A	Mar. 4, 8.00 p.m.	6	0.2	0.44	80.0
	9.00 "	7			76.0
	10.00 "	8			68.6
	1918				
B*	Mar. 13, 11.30 a.m.	6			71.0
	12.30 p.m.	7			58.5
	3.30 "	10			53.7
	6.30 "	13			46.2
	7.30 "	14			45.5
	8.30 "	15			40.8
	9.30 "	16			37.2
	1920				
C	Apr. 29, 10.00 a.m.	12	1.8	1.34	58.2
	11.00 "	13	1.8	1.34	55.8
	12.00 m.	14	1.8	1.34	54.6
	1.00 p.m.	15	1.9	1.37	53.0
	2.00 "	16	2.0	1.41	50.0
	3.00 "	17	1.8	1.34	49.5
	4.00 "	18	2.0	1.41	47.1
	5.00 "	19	2.3	1.51	44.2
D	Apr. 22, 11.30 a.m.	16			53.2
	12.30 p.m.	17			50.7
	2.30 "	19	1.9	1.37	45.0
	3.30 "	20	2.0	1.41	42.3
	4.30 "	21	2.6	1.61	39.5
	5.30 "	22	3.2	1.78	37.7
	7.30 "	24	5.0	2.23	33.4
	8.30 "	25	6.4	2.52	34.3
	9.30 "	26	6.9	2.62	35.8
	10.30 "	27	5.5	2.34	37.6
E	Apr. 30, 4.00 p.m.	26	5.3	2.31	37.5
	5.00 "	27	4.3	2.07	38.1
	7.00 "	29	2.7	1.64	40.4
	8.00 "	30	2.3	1.51	38.6

* These observations were made on a larger fermentation of 24,000 gallons of mash.

increasing rapidity of CO_2. Beijerinck (2) does not speak of the composition of the gas produced at the start of the fermentation but of the total gas produced during the early hours of the fer-

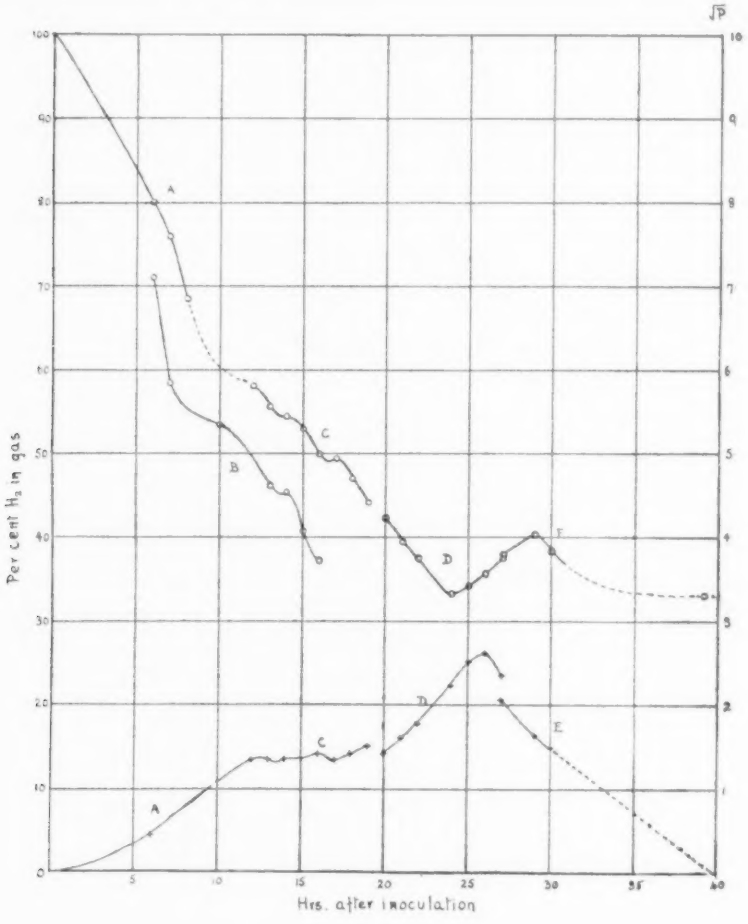

FIG. 2.

mentation. His observations could be made only at intervals of several hours owing to the fact that he was working with slower fermentations of small volumes of wort.

Further points of interest in the results obtained are as follows: After a period of about 5 hours the percentage of H_2 in the gas falls more rapidly during several hours, while during this time the rate of gas production is increasing. These changes are apparently brought about by the start or acceleration of some reaction by which CO_2 is produced. As we approach the period during which the acidity of the medium is at the maximum the composition of the gas does not change so rapidly. Furthermore it has been observed on numerous occasions that when, at the point of maximum acidity of the mash, the rate of gas production diminishes temporarily, the percentage of H_2 contained in the same remains constant or slightly increases. Attention is called to Section C of the curves in Fig. 2 which show clearly how on two occasions during the same fermentation these changes occurred. They are an indication of the complexity of the gas-producing system.

When the rate of gas production is once more increasing rapidly the percentage of H_2 in the gas falls at a uniform rate. Later, when the rates of production of acetone and butyl alcohol begin to diminish, and the rate of gas production is approaching its maximum, the amount of H_2 in the gas rises during 4 to 6 hours from 33 to 34 per cent to 38 to 40 per cent. Towards the close of the fermentation when gas production is falling rapidly to zero the percentage of H_2 in the gas falls once more. It would appear that just previous to the end of the fermentation the gas contains approximately 33 per cent.

Series III.

A number of experiments have been performed in order to determine the volume and composition of the total gas produced during the fermentation of a known weight of maize meal. A small volume of mash of known concentration was inoculated and allowed to ferment to completion in a water bath maintained at 36°C. The gas produced was collected and analyzed. It has been found that from 1 gm. of meal approximately 350 cc. of gas are produced, and that the gas is composed of 47.5 parts of H_2 and 52.5 parts of CO_2 by volume.

The results from this investigation are in close agreement with those of Beijerinck for *Bacillus butylicus*; they differ in several important points from those of Gill (4) and Reilly and his collaborators (5) whose observations, as in the case of the writer's, were made on fermentations by the Weizmann organism.

The Biochemistry of Acetone and Butyl Alcohol Production.

With the assistance of these results and those contained in an earlier communication (1) we shall now discuss briefly the biochemistry of the fermentation. Reilly and others (5) have recently presented in greater detail the view that acetone is formed from acetic acid. They consider that this is brought about by a condensation of the acid molecule with the production also of CO_2 and H_2O. It is shown that such a reaction is theoretically possible from the chemical standpoint and in accordance with certain experimental observations which have been made. The principal experimental evidence upon which this theory is based, namely the increase in the yield of acetone when acetic acid is added to the mash, when considered with observations made in this laboratory of the influence on the fermentation exerted by acids of the same series, requires, however, a different interpretation. Furthermore the results of different investigators have shown that the production of gas, H_2 and CO_2, and the formation of acetone and butyl alcohol are intimately connected, and cannot be considered as entirely separate portions of the biochemical mechanism. The alcohols have been shown to be formed by the reduction of the corresponding acids, possibly by the addition of H_2 derived from some closely associated reaction. These facts appear to suggest that the formation of H_2 is closely connected with the production of acetone. It is felt that the scheme suggested by Reilly is weakened by the fact that acetone and butyl alcohol production have been considered apart from gas production, and as a result the source of H_2 has been relegated to some unknown associated reaction.

Newman (7) has recently brought forward a biochemical mechanism for the fermentation. He considers the first reaction to be the production of butyric acid from glucose in accordance with the following equation.

$$C_6H_{12}O_6 = C_3H_7.COOH + 2\,H_2 + 2\,CO_2$$

Acetic acid is then formed by the further oxidation of butyric acid:

$$C_3H_7.COOH + O = CH_3CHOH.CH_2.COOH$$
$$CH_3.CHOH.CH_2.COOH + H_2O = 2\,CH_3.COOH$$

Acetone is formed by the oxidation of butyric acid and butyl alcohol by its reduction.

The experimental results contained in this communication show that gas production at the commencement of the fermentation is not in accordance with Newman's first equation. His scheme necessitates a gradual fall in the H_2 content of the gas from an initial content of 50 per cent. Furthermore, the comparative evidence from the investigation of the oxalic acid fermentation of glucose by *Aspergillus niger* (8) and of xylose by silage bacteria (9) supports our earlier conclusion that the two organic acids are formed by oxidation and a break in the glucose molecule. It is also necessary to point out that Newman, in order to facilitate oxidation, introduces oxygen into his scheme without any indication as to its source. We are, however, considering the life processes of an organism which will not function in the presence of free oxygen, and consequently the essential problem is that of anaërobic respiration. Jost (10) has summarized earlier research and discussed in greater detail the nature of the problem.

The following scheme is suggested as being in harmony with (*a*) experimental observations, (*b*) the requirements of the organism, and (*c*) the conditions obtaining during the fermentation. It has been previously shown that the first two stages of the fermentation may be represented by the following equations.

(1) $$(C_6H_{10}O_5) + x\,H_2O = x\,C_6H_{12}O_6$$
Starch — Glucose

(2) $$C_6H_{12}O_6 = C_3H_7.COOH + CH_3.COOH + [O_2]$$
Glucose — butyric acid — Acetic acid

The oxygen produced during the second reaction cannot be detected by ordinary chemical methods and therefore must be used up in closely associated reactions by which oxidation is brought about. It has been shown that acetone production commences very early in the fermentation and previous to the

start of butyl alcohol production (1). The gas given off at the commencement is pure H_2, but immediately a gradual and increasing dilution by CO_2 occurs in the gas being produced. From this evidence we conclude that acetone is formed by the oxidation of butyric acid in accordance with the following equations.

(3) $$2\,C_3H_7.COOH + O_2 = 2\,CH_3.CO.CH_2.COOH + 2\,H_2$$
n butyric acid — Acetoacetic acid

(4) $$CH_3.CO.CH_2.COOH = CH_3.CO.CH_3 + CO_2$$
Acetoacetic acid — Acetone

Acetoacetic acid has not been identified in this laboratory, but owing to the nature of the biochemical system this failure to isolate one of several intermediate compounds does not rule out our interpretation of other experimental evidence. It is of interest to observe also that Raistrick and Clark (8) have recently stated that from unpublished results they conclude that acetoacetic acid is an intermediate compound in this fermentation. Attention also is called to the work of Witzemann (11) who has obtained acetone as a result of the β-oxidation of butyric acid by means of H_2O_2.

The formation of butyl alcohol, and to a much smaller degree of ethyl alcohol, takes place by the reduction of the corresponding acid, in accordance with the following equations.

(5) $$C_3H_7.COOH + 2\,H_2 = CH_3.CH_2.CH_2.CH_2OH + H_2O$$

(6) $$CH_3.COOH + 2\,H_2 = CH_3.CH_2OH + H_2O$$

Apparently this reduction is not brought about by the activity of specific enzymes, owing to the fact that when propionic acid is added to the mash it is partially reduced to propyl alcohol (1).

It is necessary also to account for the following experimental data: (*a*) the disappearance of a larger amount of acetic acid than can be accounted for by the amount of ethyl alcohol produced during the fermentation; (*b*) the volume of H_2 and CO_2 given off which cannot be accounted for by the activity of reactions previously mentioned in this scheme; and (*c*) the consumption of $[O_2]$ made available by the production of butyric acid which is not further oxidized to acetone, but reduced to the alcohol. These

problems and the biochemistry of the postfermentation period are being investigated and will be considered in a further communication.

SUMMARY.

1. An investigation of the acetone and butyl alcohol fermentation of starch has been continued. Results are given of experiments in connection with the following problems.

(*a*) Changes in the composition of the gas produced during the fermentation period.

(*b*) The volume and composition of the gas produced by the fermentation of a known weight of maize.

2. The biochemical mechanism of the fermentation is discussed in the light of these results and those contained in an earlier communication.

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THE EFFECT OF WHOLE BLOOD TRANSFUSION ON THE CHOLESTEROL CONTENT OF HUMAN SERUM IN PERNICIOUS ANEMIA.

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Among the substances which inhibit the hemolytic action of saponin (1–3), tetanolysin (4), pneumococcus hemotoxin (5), bile salts (6), and oleates (7, 8), cholesterol has been shown to assume a position of considerable importance. This general antihemolytic property of free cholesterol thereby derives for itself a close relation to the study of pernicious anemia, in which disease the marked reduction of erythrocytes and hemoglobin is of unknown etiology.

With the lowering of the red blood cell count and the hemoglobin in pernicious anemia, it has been demonstrated that cholesterol is also invariably lower than normal. Thus in three cases of pernicious anemia Csonka (9) found that the cholesterol content of the blood was lower than that of a normal individual. Bloor (10) reported that of twelve cases of pernicious anemia, the total cholesterol varied from 140 to 173 mg. averaging 156 mg. per 100 cc. of plasma. Like figures are reported by Denis (11), who also found an average of 156 mg. per 100 cc. of blood in twelve cases of this disease.

The cholesterol content of the erythrocytes is known to exist almost entirely in the free form (12), there being little or no cholesterol ester present. Since this free cholesterol as stated above, and not cholesterol ester, has been shown to possess the antihemolytic property, its presence in the corpuscles, as such, apparently constitutes an immediate and appropriate mechanism of protection against the hemolytic substances brought into contact with the corpuscles in the blood. A replenishing source of free cholesterol is present in the plasma, which normally contains 25 to 35 per cent

of the total cholesterol as free cholesterol (13). This free cholesterol is probably taken up by the corpuscles through the activity of their cholesterase (14).

It would seem desirable to determine the variation in the free cholesterol but since a normal relation is maintained in the plasma in pernicious anemia between the free and cholesterol ester (10), variations in the total cholesterol would indicate variations in the free form.

Because of the variation below normal of this antihemolytic substance in the blood of patients suffering from pernicious anemia, it was of particular interest to note the effect of the addition to the serum of the patient of a quantity of blood, the serum of which contained a much greater amount of cholesterol. Denis (11) has stated that in three cases markedly improved by blood transfusion the cholesterol content of the blood was not proportionately increased.

Through the courtesy of Dr. G. C. Weil of Mercy Hospital, a series of cases presenting the cardinal symptoms and signs of pernicious anemia was available for study. The blood pictures of these cases were those of pernicious anemia of severe grade with the typical alteration in the appearance of the erythrocytes, as well as the diminution of their number and corresponding reduction of hemoglobin percentage.

Blood was transfused directly by a modified Unger method. Blood from the recipient and donor was obtained immediately before, and from the recipient from 3 to 5 minutes after, transfusion.

The results of the analyses of the sera from these specimens of blood, using the method of Bloor (15) compared with the hemoglobin estimations and erythrocyte counts are shown in Table I.

In all these cases there is shown an increase in the total cholesterol content of the serum of the recipient following the transfusion. This increase varied from 5 to 34 mg. averaging 18 mg. per 100 cc. of serum. That the increase is only a temporary one is shown by the return of the cholesterol figure to approximately that immediately preceding the transfusion. The variability of the increase is probably dependent upon the degree of admixture of the blood of the donor with that of the recipient, and upon the

TABLE I.

Case No.	Date.	Blood examination.		Total cholesterol per 100 cc. of serum.	Amount of whole blood transfused.
		Hemoglobin.	Erythrocytes.		
	1920	*per cent*		*mg.*	*cc.*
1	Jan. 26	48	1,960,000		
	" 30			R. B.* 181	
				R. A. 227	450
				D. B. 308	
	Feb. 1			R. B. 148	500
	" 11			R. B. 135	
				R. A. 168	500
				D. B. 308	
	" 20			R. B. 183	260
	" 24	62	3,240,000		
	Mar. 8			R. B. 133	
				R. A. 145	400
				D. B. 333	
	" 11	65	3,750,000		
	Gradual marked improvement.				
2	Jan. 20	40	1,320,000		
	" 26				500
	" 29	45	2,210,000		
	Feb. 2			R. B. 131	
				R. A. 148	400
				D. B. 250	
	" 6	60	2,290,000		
	" 9			R. B. 139	
				R. A. 185	340
				D. B. 297	
	" 17	68	3,950,000		
	" 18			R. A. 153	
				D .B. 227	340
	" 24	75	3,470,000		
	Mar. 3			R. B. 137	
				R. A. 138	500
				D. B. 268	
	" 11	78	3,650,000		
	Marked clinical improvement.				

* R. B., recipient before transfusion. R. A., recipient after transfusion. D. B., donor before transfusion.

TABLE I—*Continued.*

Case No.	Date.	Blood examination.		Total cholesterol per 100 cc. of serum.	Amount of whole blood transfused.
		Hemoglobin.	Erythrocytes.		
	1920	*per cent*		*mg.*	*cc.*
3	Jan. 27	64	2,300,000		
	Feb. 2			R. B. 193	
				R. A. 227	500
				D. B. 423	
	" 7	68	3,000,000		
	" 17	50	1,410,000		
	" 20			R. B. 195	
				R. A. 203	440
				D. B. 273	
	" 24	55	2,370,000		
	General condition improved.				
	1919				
4	Nov. 5	22	900,000		
	" 14			R. B. 123	
	" 18	55	1,810,000		
	" 28			R. B. 143	
	Dec. 1	62	2,680,000		
	" 9			R. B. 163	400
	" 11	65	2,330,000		
	" 17			R. B. 211	400
	1920				
	Jan. 9				320
	" 16				440
	" 19	80	3,440,000		
	Marked improvement in general condition at this time.				
	Feb. 10	60	1,770,000		
	" 25			R. B. 130	
				R. A. 141	500
	" 28	48	2,240,000		
	Mar. 3			R. B. 133	
				R. A. 152	
	" 8	45	1,890,000		
	" 10			R. B. 129	
				R. A. 153	420
				D. B. 166	
	" 24	56	1,950,000		
	" 27			R. B. 124	
				R. A. 135	500
				D. B. 238	

TABLE I—*Continued.*

Case No.	Date.	Blood examination.		Total cholesterol per 100 cc. of serum.	Amount of whole blood transfused.
		Hemoglobin.	Erythrocytes.		
	1920	*per cent*		*mg.*	*cc.*
	Apr. 8	40	2,050,000		
4	" 17	35	1,390,000		
	" 19			R. B. 93	
				R. A. 97	500
				D. B. 227	
	General condition continued improved.				
5	Jan. 24	40	1,310,000		
	" 28			R. B. 128	500
	Feb. 18			R. B. 139	
				R. A. 135	500
				D. B. 403	
	" 26	50	2,000,000		
	Mar. 8			R. B. 121	
				R. A. 138	300
				D. B. 320	
	" 24	53	1,280,000		
	Apr. 1				300
	" 12	30	1,240,000		
	" 13				400
	" 15	35	1,570,000		
	" 26				400
	May 7				460
	" 16				420
	" 26			R. B. 104	
				R. A. 142	500
				D. B. 373	
	" 27	48	2,780,000		
	General condition remained fair.				
	1919				
6	Nov. 12	22	1,160,000		
	" 29	40	1,370,000	R. B. 123	
				R. A. 157	420
	Dec. 3	52	2,670,000		
	" 16			R. B. 140	400
	" 17	65	2,780,000		
	1920				
7	Mar. 23	45	1,720,000	R. B. 154	
				R. A. 153	420
				D B 438	

TABLE I—*Concluded.*

Case No.	Date.	Blood examination.		Total cholesterol per 100 cc. of serum.	Amount of whole blood transfused.
		Hemoglobin.	Erythrocytes.		
	1920	*per cent*		*mg.*	*cc.*
7	Apr. 1	50	2,120,000		
	" 6	47	1,850,000		
	" 7			R. B. 156	
				R. A. 150	400
				D. B. 240	
	" 19			R. B. 164	
				R. A. 166	540
				D. B. 231	
	May 1	45	1,710,000		
	" 6	35	1,800,000		
	" 17			R. B. 166	
				R. A. 182	400
				D. B. 183	
	" 24	32	1,690,000		
	" 27	33	1,370,000		
	June 4			R. B. 161	
				R. A. 187	400
				D. B. 250	
	June 11	Death. Pernicious anemia.			
8	Jan. 5				400
	" 8				500
	" 15	55	2,530,000		
	" 16				500
	" 20	63	2,690,000		
	" 28	70	2,450,000		
	" 30			R. B. 147	
				R. A. 152	500
				D. B. 347	
	Feb. 11			R. B. 173	
				R. A. 179	280
				D. B. 316	
	" 17	65	2,780,000		
	" 23			R. A. 128	
				D. B. 304	280
	Mar. 11	Death after a gradual increase in severity of symptoms.			

length of time which elapses before the sample of blood following the transfusion is taken for analysis from the vein of the recipient into which the blood has been infused.

In the first five cases which showed distinct clinical improvement following repeated transfusion, the cholesterol content of the serum was not progresively increased. On the other hand in those cases having a fatal termination the cholesterol content was not proportionately lowered and at times not at all.

In Case 4, the first series of isolated analyses, where blood specimens were taken before transfusion, a progressive increase is seen in the cholesterol content of the serum, paralleling a distinct improvement in the clinical condition of the patient and a marked increase in the red blood cell count and hemoglobin percentage. This single instance of a relative increase in the cholesterol content of the serum with distinct clinical improvement and marked increase in erythrocytes and hemoglobin, may have occurred at a time when the patient was enjoying a remission in the course of the disease. This improvement was continued, but a lapse of treatment for approximately 2 months during this period brought about a considerable turn for the worse in the blood picture, with a return of the cholesterol to the previous low figure. This would seem to indicate that there is a close relation between the cholesterol content of the serum and the erythrocyte content and hemoglobin percentage. This relation is further borne out by a study of the other cases which shows that, in all but Cases 1 and 2, the cholesterol content bears some relation to the blood picture in holding to a constant low level with the continued depression of the red blood cell count and the hemoglobin percentage.

It is interesting to note that in Cases 1 and 2, with a marked improvement in the blood picture and clinical condition, and in Case 4, with continued clinical improvement and a practically constant blood picture, the cholesterol content is even slightly lower, indicating perhaps an increased utilization of this substance against the toxic elements causing hemolysis in the disease.

CONCLUSIONS.

1. Repeated whole blood transfusion does not appreciably alter the total cholesterol content of the serum in pernicious anemia.

2. The immediate increase in cholesterol in the serum of the recipient, following transfusion of whole blood, is dependent upon the degree of admixture of the blood from the donor with that of the recipient and upon the time which has elapsed after the transfusion, when the specimen of blood from the recipient is obtained.

3. The cholesterol content of the serum varies relatively with the degree of anemia and the hemoglobin percentage of the blood.

4. The cholesterol level in the serum of patients with pernicious anemia may be an index of its utilization against hemolytic substances present in the body in this disease. The relative depression of cholesterol in the more severe cases indicates an increased demand for the neutralization of these toxic substances.

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USE OF SYNTHETIC DIETS IN THE GROWTH OF BABY CHICKS.*

A STUDY OF LEG WEAKNESS IN CHICKENS.

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Plates 1 and 2.

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In the rearing of baby chicks in confinement a difficulty serious and obscure in its etiology is the one characterized by poultrymen as "leg weakness." This trouble usually develops in 4 to 6 weeks after hatching, but we have seen it show itself 4 to 6 weeks later. The principal symptoms shown by the bird are first an unsteady gait, developing into difficulty of locomotion with a tendency to remain squatted a good part of the time; a pronounced ruffled condition of the feathers; an anemic condition of the wattles and comb; and a swelling of the leg joints, which is sometimes permanent. A loss of appetite accompanies these conditions and usually death follows suddenly. Postmortem examination has not revealed any pronounced and characteristic changes. With some individuals there is compaction of the feces in the cecum, while with the others the cecum content is fluid and gassy. These latter observations may be related to the postmortem time of examination. With some individuals the liver was found abnormal in color, being pale yellow, but this condition was not found in all birds suffering from leg weakness and should not be considered an invariable occurrence.

Poultrymen have explained leg weakness as due to lack of exercise, overfeeding, or absence of green feed, and their remedy

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has been to feed light, provide a suitable scratch, and if possible feed some green feed. These practices will generally lead to an eradication of this trouble, but just which factor is the primary one in effecting a cure has not been clearly defined.

Drummond[1] experienced trouble in the use of the baby chick as an experimental animal in confinement. He believed that they could not be reared under such conditions, or at least expressed the view that there were very special conditions necessary for such rearing and not understood at the time when he wrote. Funk[2] was also familiar with the difficulties experienced in rearing baby chicks at all seasons of the year and under laboratory conditions. Osborne and Mendel,[3] in attempts to use the chicken as an experimental laboratory animal, experienced similar difficulties with leg weakness. They succeeded, however, in rearing a number of chickens taken either at hatching time or at 3 weeks of age on a ration of natural materials, but which was fortified with a plentiful supply of roughage in the form of paper pulp. The rations were varied, but corn gluten, cotton-seed flour, soy bean flour, casein, protein-free milk, butter fat, starch, lard, and ferric citrate entered into the diet.

This observation, involving the recognition of the very great importance of a roughage factor in the diet of baby chicks, confirms our own experience in work with this animal under confinement and we believe may be taken as directly responsible for the prevention of leg weakness in chickens. We have worked with a variety of roughages, some of which were potent as protectives while others were not. Suspicion that this trouble in chickens might be scurvy led us to use orange juice in a number of cases as a curative, but the results were too varied and too distinctly negative to warrant the idea of a similarity in etiology between leg weakness and scurvy. The entire trend of our work is in confirmation of the observations of Osborne and Mendel that leg weakness is related to overfeeding, with a low amount of indigestible ballast in the ration and not to a lack of green feeds, exercise, outdoor air, or excessive feeding directly. Undoubtedly the latter is effective in hastening the development of

[1] Drummond, J. C., *Biochem. J.*, 1916, x, 77.
[2] Funk, C., *J. Biol. Chem.*, 1916, xxvii, 3.
[3] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiii, 433.

leg weakness, because in the presence of plenty of roughage excessive feeding cannot take place. Possibly green feeds with their abundant supply of vitamines and some roughage are an unconscious cure of a sluggish intestine, but the same results can be obtained with an inert ballast such as paper, dirt, or charcoal.

EXPERIMENTAL.

As early as 1917 we began a study of the nutritive factors involved in the rearing of baby chicks. We used synthetic rations such as had been successfully employed in the rearing of the rat. With a ration composed of 18 parts of casein, 69.3 of dextrin, 5 of butter fat, 3.7 of salts, 2 of agar, and 2 of yeast there was complete failure in growth with the baby chick. On this ration a rat could be successfully reared. In all our experiments the casein was purified; the yeast was in dried powder form procured from a commercial brewing company; the butter fat was purified as customary for incorporation in the ration of purified food materials; the salt mixture consisted of

	gm.
NaCl	0.173
$MgSO_4$ (anhydrous)	0.266
$NaH_2PO_4H_2O$	0.347
K_2HPO_4	0.954
$CaH_4(PO_4)_2H_2O$	0.540
Iron citrate	0.118
Ca lactate	1.300
KI	Trace.

Started on this ration directly after hatching and at 39 to 40 gm. weight, baby chicks would grow for a short time, that is up to 50 or 60 gm., but would soon begin to lose weight and death would follow at the end of 3 to 5 weeks. Even increasing the yeast in the ration to 4 per cent thereby providing more water-soluble vitamine did not materially improve the ration. These animals had been on a shavings litter and had received limestone grits in a separate dish. Their rations had been fed as a mash and distilled water was always used.

In a later series of experiments where we used a synthetic ration such as described above, but more liberal in its content of butter fat (10 per cent) and yeast (5 per cent) and where the

animals had the run of a cut wheat straw litter, much better growth of baby chicks for 5 to 6 weeks was secured, but leg weakness soon developed and terminated the experiment. When the ration, undiluted with roughage, is fed as a mash there is no guarantee that chicks will eat enough straw provided by a cut straw litter to protect themselves fully against leg weakness.

In another series of observations, always involving six chicks in each experiment, we increased the butter fat to 15 per cent and

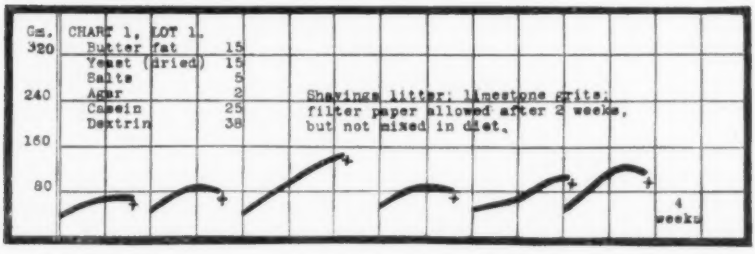

CHART 1. By merely presenting torn up filter paper to these baby chicks the development of leg weakness was not prevented. They ate some of the paper.

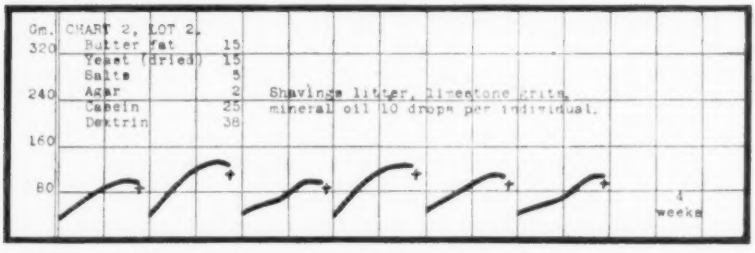

CHART 2. Mineral oil was without effect in preventing the development of leg weakness in chickens.

the yeast to 15 per cent of the ration. In all our experiments either Rhode Island Reds or White Leghorns were used. The ration was fed dry and as a mash with limestone grits. The litter was from shavings. After 2 weeks of this ration several additions were made in the hope of warding off leg weakness. In one case we added filter paper (see Chart 1). The paper was torn up by hand and thrown into the cage, but not incorporated in the diet. They ate some of it, but not enough to prevent

trouble. To another group (Chart 2) we gave mineral oil daily, at the rate of 10 drops per individual. This treatment was without effect. In Chart 3 are shown the results where the laxative, phenolphthalein, was used at the rate of 0.01 per cent of the ration. This addition gave no beneficial reaction and no better results than were secured by the use of mineral oil. Hull and Rettger[4] had reported the successful use of lactose as a means of reducing putrefaction in the intestine of the rat with a change in

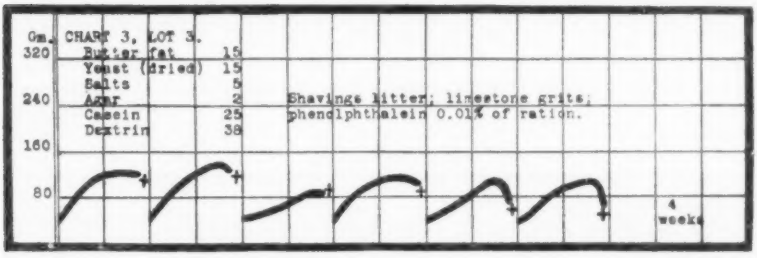

CHART 3. The use of phenolphthalein, a laxative, was not a successful measure for the prevention of leg weakness.

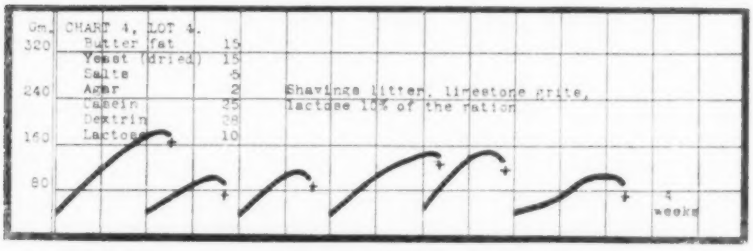

CHART 4. Lactose as 10 per cent of the ration did not prevent the development of leg weakness.

the character of the intestinal flora. If leg weakness is associated with a sluggish intestine and slow removal of waste then the possibility of curative results from the use of lactose is apparent. We incorporated this sugar in the ration at the rate of 10 per cent. In the concentration used it had no preventive effect on the development of leg weakness (see Chart 4).

[4] Hull, T. G., and Rettger, L., *J. Bacteriol.*, 1917, ii, 47.

It had occurred to us that leg weakness might be "scurvy of chickens" and therein might rest the reasons for the successful use of green feed so generally advocated by all poultrymen. With this possibility in view we gave one lot of baby chicks, after they had been on the synthetic ration for 2 weeks, a daily allowance of crushed green cabbage leaves (Chart 5). They ate this material eagerly and probably consumed as much as would be expected from a bird of this size when allowed green materials under practical conditions. We had no better success with this lot than with those already described. This result in itself would strongly indicate that we were not dealing in the case of leg weakness with scurvy in chickens. Further confirmation of this view is afforded in later work herein reported where we administered daily the

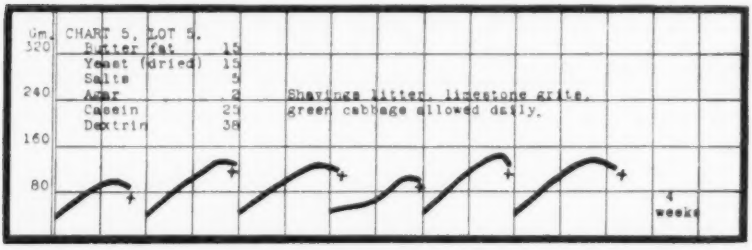

CHART 5. Green cabbage given daily did not ward off the development of leg weakness. This is a potent antiscorbutic and these results indicate that scurvy and leg weakness in chickens have not the same etiology.

classical antiscorbutic, namely orange juice, to a number of lots of chickens developing leg weakness, but without effecting cures. Possibly we delayed the termination of life by its use, but did not bring about such cures as can be accomplished with a guinea pig already seriously afflicted with the disease of scurvy.

In Chart 6 are shown the results of a direct attempt to cure leg weakness with a potent antiscorbutic, such as orange juice. Its administration daily in 1 cc. portions is a larger allowance per unit of live weight than has been used in the successful treatment of guinea pigs afflicted with scurvy. All this lot came down with leg weakness and continued to suffer from this affliction, although there was a much greater prolongation of the span of life by the use of orange juice. We never succeeded in effect-

ing complete cures or warding off the development of leg weakness by the use of orange juice when used at the rate of 1 cc. daily per individual. Further, the very fact that we finally succeeded in rearing to maturity birds whose diet was adequately diluted with some inert ballast is positive proof that we are not dealing with scurvy. In earlier work[5] with chickens, restricted to grains fortified with inorganic salts, we observed slow growth and normal maintenance for 8 months, when the experiment was terminated. These results are given as further evidence that the chicken is not supersensitive to a low antiscorbutic vitamine ingestion as is the case with the guinea pig.

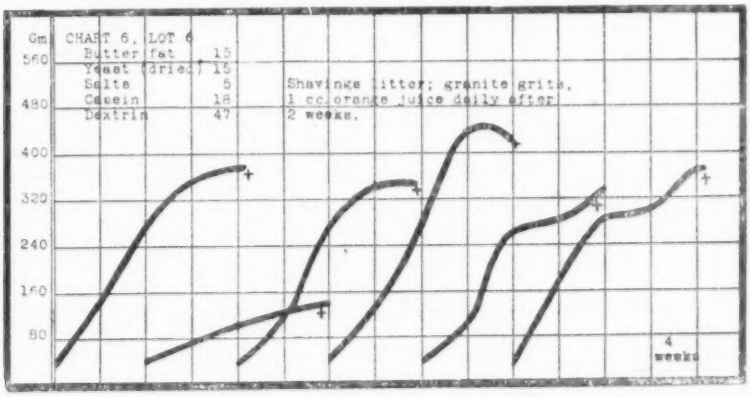

CHART 6. Leg weakness is not scurvy. Orange juice at the rate of 1 cc. daily per individual delayed death, but did not prevent the comparatively early termination of life of this group with leg weakness.

We next began a systematic study of the influence of inert materials as roughages in the ration with special attention to their nature as well as their quantity. An interesting failure is shown in Chart 7 where we used 10 per cent of filter paper in the ration. In this case the mixed nutrients were hand-mixed with the finely rasped paper. The mixture of paper and ration was not intimate enough to prevent picking out by the birds. They picked out the food ingredients leaving behind the paper. Leg

[5] Hart, E. B., Halpin, J. G., and McCollum, E. V., *J. Biol. Chem.*, 1917, xxix, 57. Hart, E. B., Halpin, J. G., and Steenbock, H., *J. Biol. Chem.*, 1917, xxxi, 415.

weakness developed after 5 weeks restriction to this diet. After this group developed leg weakness we began the daily administration of orange juice but without curative effects. Contrast these results with those shown in Chart 12 where the paper was reground with the ration and where no orange juice was added. In this case complete success in rearing was attained.

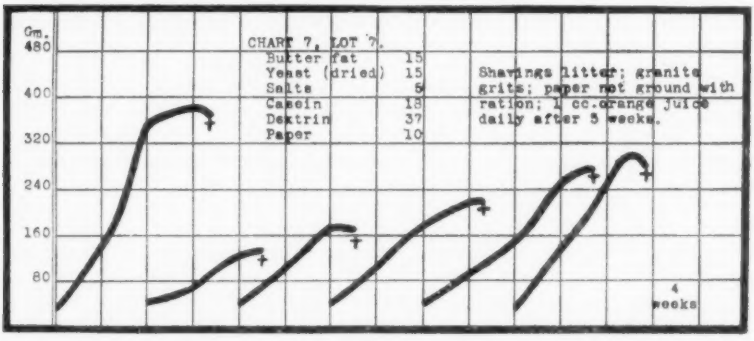

CHART 7. Paper mixed but not ground with the ration did not prevent the development of leg weakness. The birds did not eat enough of the paper. Administration of orange juice showed no remedial effects.

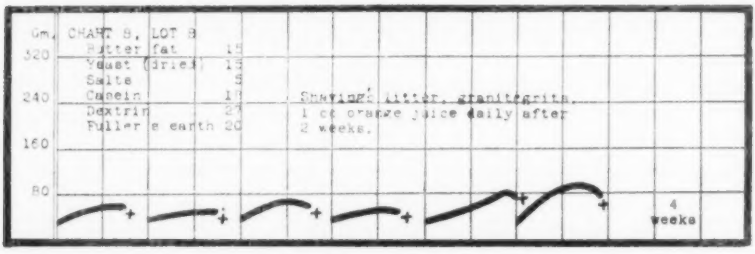

CHART 8. Fuller's earth is not a good roughage for baby chicks. Orange juice, a potent antiscorbutic, was of no help in preventing early death of the individuals in this lot.

We found fuller's earth a decidedly ineffective ballast. This colloidal siliceous material, used as 20 per cent of the diet, seemed to hasten the downward path of this group of birds. The results are shown in Chart 8. Shavings were used as litter and undoubtedly the chickens consumed some of this material, although not enough to act as an efficient protection against leg weakness.

After 2 weeks on the ration we gave orange juice at the rate of 1 cc. daily per individual, but without any help.

When we incorporated 20 per cent of charcoal in the ration with shavings as a litter we met with varied success (Chart 9). Three of the birds developed leg weakness, while three grew well, avoiding all trouble from this disease and reaching weights of 1,240, 706, and 805 gm., respectively, before being discarded. They were apparently normal in every respect. Just why we had mixed results is impossible to state, but there is a very considerable individual variation in chickens, making their use for nutrition work less satisfactory than the rat.

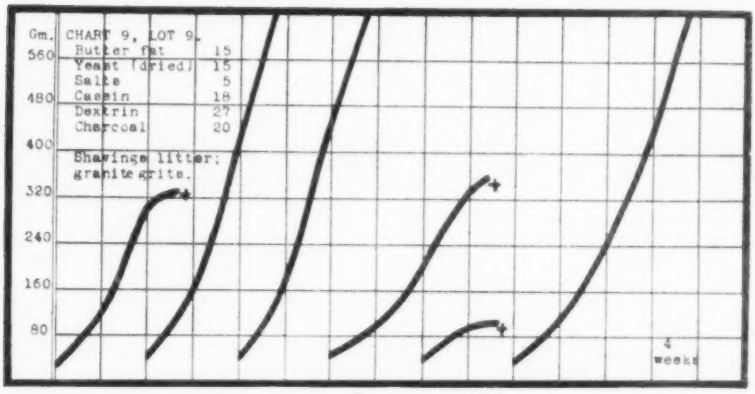

CHART 9. Charcoal was an effective roughage, but did not give us unqualified success.

The practice of turning baby chicks out on fresh upturned and unfrozen ground just as early after hatching as possible, with the time of hatching purposely regulated with reference to spring weather in order that this condition may be satisfied, is followed by poultrymen. The birds undoubtedly eat a considerable amount of this dirt; but that it is a nutritive factor of primary importance in their rearing is open to careful consideration. We incorporated into our synthetic ration 20 per cent of an air-dried garden soil and one not particularly rich in organic matter. The results can be seen in Chart 10. Two of the birds grew normally and reached weights of 1,380 and 995 gm. respectively, when discarded. They were apparently normal in every respect. Four

of the chickens in this lot did not grow well, developed leg weakness, and died at the end of 6 to 12 weeks. Orange juice was given these birds at the appearance of symptoms of leg weakness, but did not save them from an early death. It is possible that the amount of dirt fed was not well chosen for the reason that in later experiments we practically duplicated the above experiments by the use of 20 per cent of dirt on a sand litter, but had very much better success when we used but 10 per cent of dirt (*cf.* Charts 19 and 20).

All the experiments described above were conducted on shavings litter. We believe that this factor might have been the

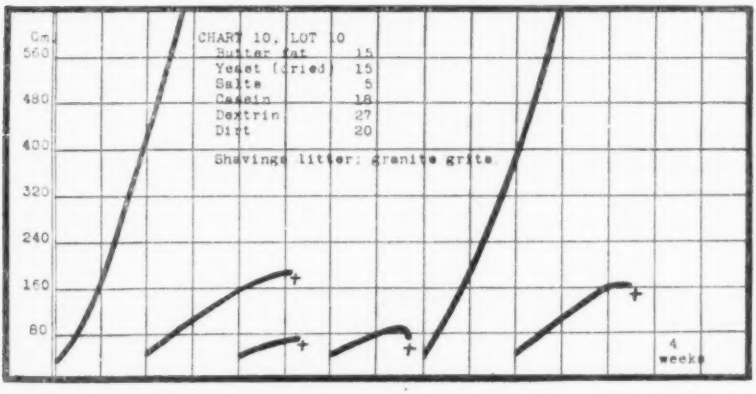

CHART 10. Dirt also was an effective roughage in some cases, but not of general success with the synthetic diet.

cause of the pronounced individual variation seen in our results. It was possible that some birds ate more of the shavings than others and thereby escaped the trouble of leg weakness. Accordingly we adopted sand as the litter, using the same synthetic rations as used in the earlier lots, whose curves of growth are displayed in Charts 6 to 10 inclusive. In these later experiments they may have eaten some of the sand, but its crystalline character as contrasted with the colloidal nature of shavings (cellulose, etc.) would probably make it very much less a disturbing factor than the shavings litter.

The rations were always fed dry or as a mash with distilled water and granite grits; they were never fed as scratch. The

roughage materials used were in the proportion of 10 or 20 per cent of the ration, although in the case of paper the maximum amount incorporated was 10 per cent and the minimum 5 per cent of the ration. The results secured with these rations are shown

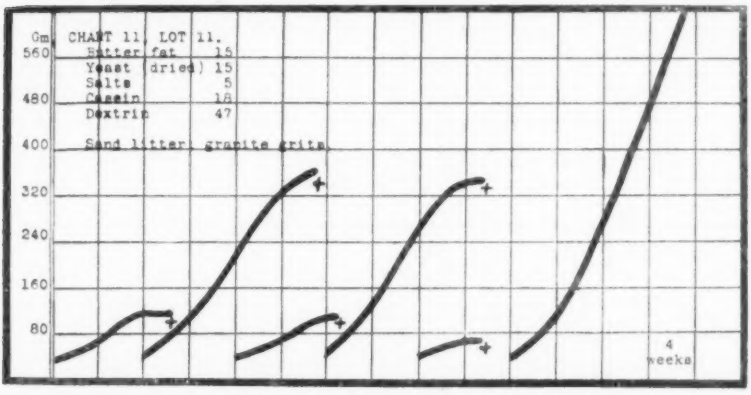

CHART 11. On sand litter and no roughage all the birds but one developed leg weakness.

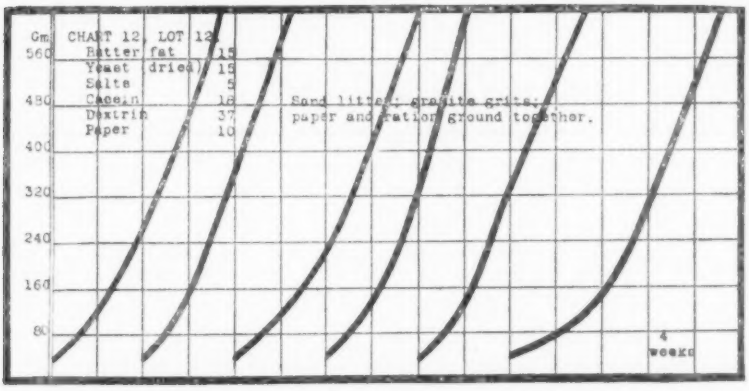

CHART 12. With 10 per cent of paper ground with the ration our most successful rearing of baby chicks on a synthetic diet was accomplished.

in Charts 11 to 20 inclusive. One group of chicks, Chart 11, was fed our synthetic ration, to which, however, no roughage was added. Five of the six individuals in this lot succumbed to leg weakness, but curiously enough one animal grew well, and did not develop

this trouble, reaching a weight of 880 gm. at the end of 18 weeks, when it was discarded.

Our most successful rearing is shown in Chart 12. With 10 per cent of paper finely comminuted and ground with the ration every chick in this group grew at a nearly normal rate and remained physically sound. The weights at the end of 16 weeks varied from 480 to 710 gm., respectively. They were apparently normal when discarded at the end of 18 weeks. These results we think disclose in a qualitative way the factors necessary in the rearing of baby chicks. The quantitative relation of these factors is still to be studied. When but 5 per cent of paper

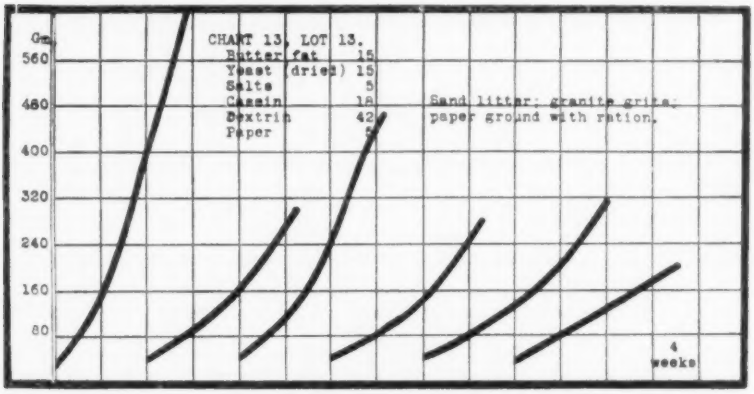

CHART 13. With 5 per cent of paper ground with the ration we were less successful than when 10 per cent was used. However, no leg weakness developed.

(Chart 13) was incorporated with the ration we did not attain the same measure of success as when 10 per cent was used. No leg weakness developed, but the rate of growth in all cases but one was slow. One bird reached a weight of 1,150 gm. at the end of 18 weeks, when the experiment was discontinued.

It is remarkable how great was the influence of the character of the ballast in these experiments. When we used 20 per cent of agar (Chart 14) in the ration, growth was practically suspended after 4 weeks and the span of life was greatly reduced. These birds showed a peculiar pulpy condition of the tissue, dropsical in character. By reducing the proportion of agar in

the ration to 10 per cent, better growth was attained than by the use of 20 per cent, although it was far below the success reached with paper, dirt, or charcoal. Three individuals from this group died from unknown causes, while the other three, all in poor condition, were discarded at the end of 13 weeks (Chart 15). None of this group had developed typical leg weakness.

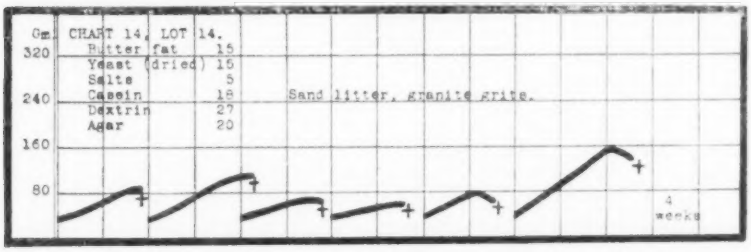

CHART 14. On a sand litter 20 per cent of agar was ineffective as a preventive of early death with the synthetic diet.

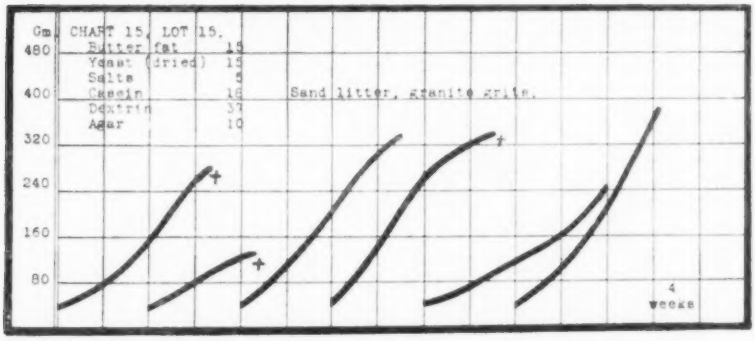

CHART 15. 10 per cent of agar gave better results than 20 per cent, but not complete success.

With 20 per cent of fullers' earth as the roughage (Chart 16) growth was subnormal and death followed early in life. This group did not develop the characteristic symptoms of leg weakness. Their appetites were enormous and their consumption records show a larger consumption of food at the end of 4 weeks than in the case of those fed the paper pulp ration (Chart 12). Probably the food was moving too rapidly through the intestine for maximum digestion and absorption.

With the sand litter and 20 per cent of charcoal (Chart 17) four of the birds grew well, reaching weights varying from 550 to 860 gm. at the end of 18 weeks, at which time they were discarded. Two birds from this group died from unknown causes, but none developed typical leg weakness. With 10 per cent of charcoal (Chart 18) the mortality was higher than where we used

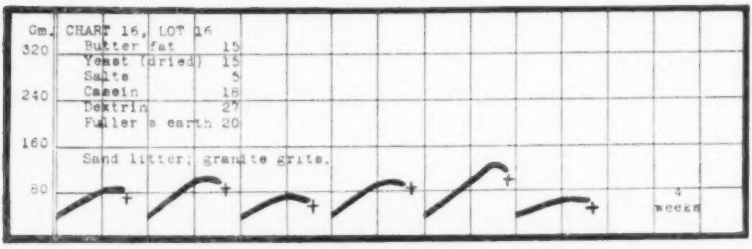

CHART 16. Fullers' earth at the rate of 20 per cent in the ration was absolutely ineffective as a preventive of early death in this group of chicks.

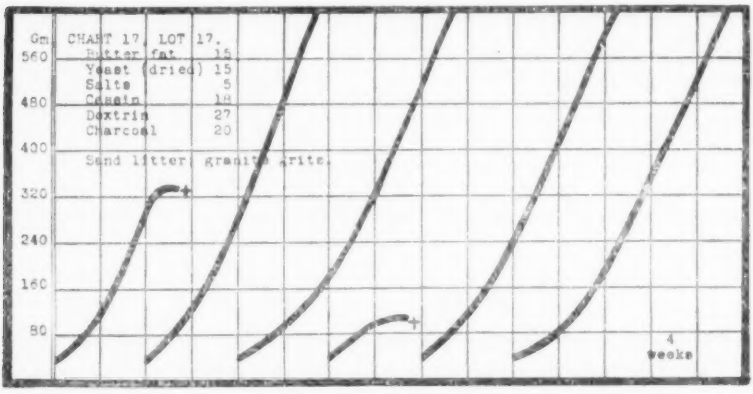

CHART 17. Charcoal at the rate of 20 per cent of the ration was less effective than 10 per cent of paper. No leg weakness developed in this group.

the higher level of 20 per cent. Two of the group reached weights of 710 and 740 gm. at the end of 18 weeks, two others grew at a much slower rate and were discarded at the end of 14 and 18 weeks respectively, while the remaining pair died at the end of 10 weeks. The cause of death was unknown, but it was not from leg weakness, with the usual symptoms.

When 20 per cent of dirt was incorporated with the ration, using the same soil as was used in our earlier experiments, we were unsuccessful in bringing any of the lot through to maturity (Chart 19). These animals did not develop typical leg weak-

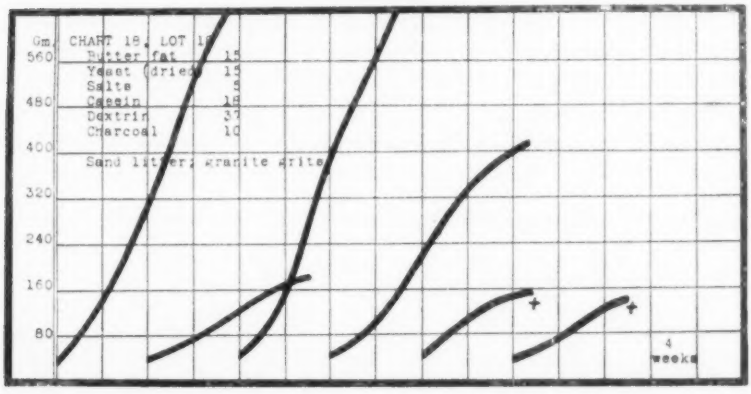

CHART 18. 10 per cent of charcoal in the ration did not lead to successful growth with all individuals, but none in the group developed leg weakness.

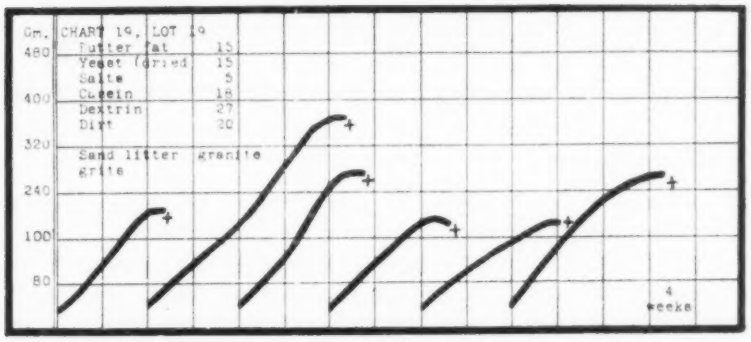

CHART 19. With 20 per cent of soil as a roughage we were unsuccessful in bringing any of the birds through to maturity.

ness, in so far as inability to walk is characteristic of that trouble, but they did show the ruffled feathers and swollen joints which are always part of the picture of leg weakness. With less dirt in the ration, 10 per cent, we were much more successful

in the rearing than where the higher level was used. Complete success was not obtained in this case (Chart 20) but four of the birds reached weights of 480 to 690 gm., respectively, in 18 weeks when they were discarded. Two from this lot died in the course of the experiment, but not from leg weakness. It is apparent that dirt itself is an effective factor in the rearing of baby chicks where the choice of the amount to be consumed is left to the bird. With a synthetic diet and under confinement the dirt was not so successful a source of ballast as was paper or charcoal.

It has been fairly well demonstrated by the work detailed above that the roughage factor when of suitable kind is the

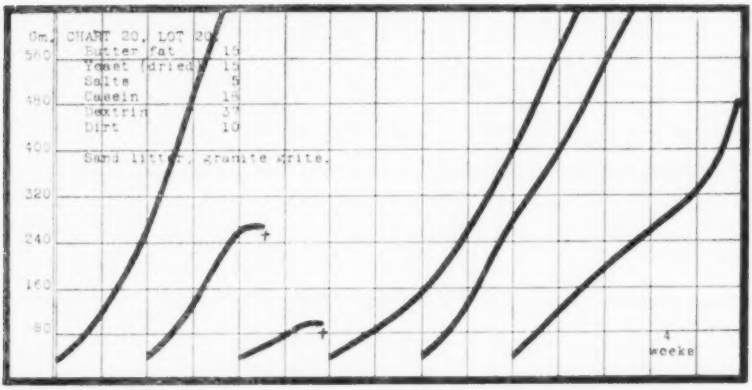

CHART 20. Incorporation of 10 per cent of soil was more effective than 20 per cent of the same material.

important one in preventing leg weakness. This malady is not due to a deficiency in one or more of the now recognized factors for successful nutrition such as energy, protein, salts, or vitamines or to a deficiency in supply of a special vitamine. Further demonstration of this fact is illustrated in Chart 21, where there was not only an abundant allowance of all the nutritive factors known at the present time, but where the exercise factor was also amply provided for. We used as scratch a grain mixture of 30 parts each of yellow corn, corn gluten, and millet; and as a mash a mixture of wheat bran 30 parts, shorts 25 parts, yellow corn meal 25 parts, and meat scraps 20 parts. Skim milk was given *ad libitum*. Green feed was daily provided by the use of

fresh, green, crushed clover. A deep litter of cut corn fodder was furnished in which the birds were compelled to work actively for their scratch feed. These animals grew at very rapid rates for 8 weeks, reaching weights varying from 332 to 417 gm. in that time. At the end of that time they all began to develop leg weakness with varying degrees of intensity. There were two factors contributing to leg weakness in the lot; first, they were overfed a highly concentrated diet and second, they did not eat enough of the roughage available and in consequence a dilution of the diet was not secured. Had the roughage been incorporated in adequate quantity with the grains and concentrates

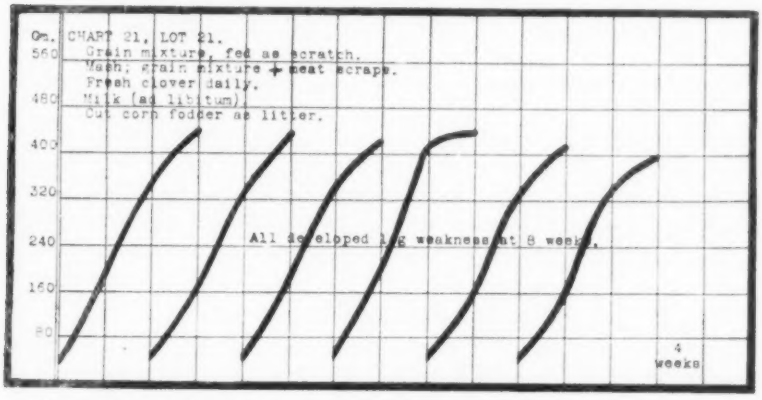

CHART 21. With all known nutrient factors present and with exercise and green feed furnished we may have leg weakness develop. The absent factor was an adequate consumption of roughage.

thereby compelling a lower consumption of absorbable nutrients probably no trouble would have resulted.

Again, as an illustration of the necessity of an intimate incorporation of the roughage, at least with synthetic diets to be used in the study of the nutrition of chickens, Charts 22 and 23 are added. In one instance we provided a cut oat straw litter and in addition mixed into the ration 10 per cent of cut oat straw. The straw was cut in lengths of approximately $\frac{1}{4}$ to $\frac{1}{2}$ inch. Leg weakness developed with three of the birds in 6 to 18 weeks and terminated their lives. The other 3 also developed this trouble, but in milder form, and grew at a slower rate of 15 to 16 weeks

when they were discarded. It is apparent that unless this class of animals will voluntarily eat the roughage in plentiful quantity, merely having it present and trusting to a haphazard consumption is to invite a high mortality. Further, it is altogether probable that the roughage should either be practically inert and lacking in taste, or should be one of an attractive palatability.

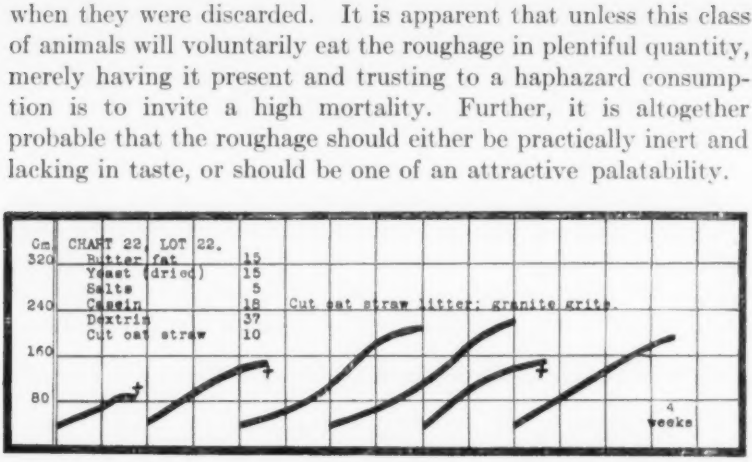

CHART 22. A failure to rear baby chicks when the roughage can be picked out and is not consumed. In this case cut oat straw served as the roughage.

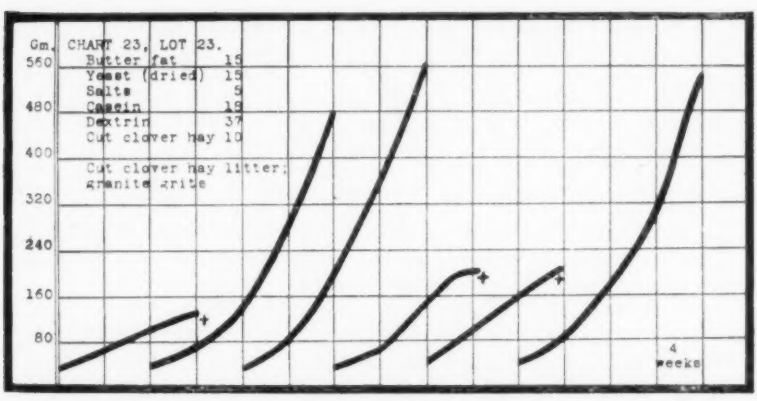

CHART 23. Another failure to rear baby chicks where the roughage is not intimately a part of the ration or so attractive in palatability as to invite consumption.

In Chart 23 are shown results secured with dry, cut clover hay used as a litter and also incorporated in the synthetic ration in the proportion of 10 per cent. Leg weakness developed in three of the birds of this lot and with such seriousness as to result in death at the end of 12 weeks. The other three birds made

slow initial growth and also developed mild symptoms of leg weakness. From this early attack of leg weakness they apparently recovered at the end of 12 weeks and showed a normal condition. The coarseness of the roughage used in these latter experiments allowed picking out which no doubt was the determining factor in both rations made with cut straw and cut clover. This would explain individual variations such as observed here, those birds getting on better when they did not separately consume the concentrated food material, but ate the entire mixture, including the roughage.

DISCUSSION.

Our results on the study of the use of synthetic diets for the rearing of baby chicks points toward the primary importance of an indigestible ballast in their ration. Why we should have such variations in the effectiveness of these materials used is impossible to state. Possibly agar failed because it may have been digested in the tract of this species, or it is possible that as a gel in the intestine it was less effective as an absorbent than charcoal, dirt, or paper; we did have some success with it at a level of 10 per cent. Fullers' earth is of a pasty character and was very inefficient as a ballast; because of its effectiveness as an absorbent it is possible that it prohibited the full reabsorption of certain essential nutrient factors, thereby hastening death. But speculation as to how these materials acted in the tract is unprofitable.

We questioned whether there might be a direct correlation between leg weakness and an abnormal permeability of the intestinal wall, believing that herein may be found the real explanation for the prevention of this trouble with an effective roughage, in that it acts as an absorbent and ready means for the elimination of toxic materials generated in the tract. With this end in view a number of precipitin reactions was made on the blood of chickens suffering from leg weakness. The precipitin tests were carried out according to the technique of Kolmer and were made for us by Dr. P. W. Boutwell of this laboratory.[6] The solu-

[6] Kolmer, J. A., A practical text-book of infection, immunity and specific therapy, Philadelphia and London, 1915.

tion of the protein against which it was thought that the animal might be sensitized was prepared as follows: 20 gm. of the ground feed (oats, corn, etc., as the case might be) were shaken up with 200 cc. of an 0.85 per cent NaCl solution and allowed to stand 1 to 2 hours. It was then filtered through a fluted filter and finally through a Berkfeld filter. This gave a perfectly clear solution which foamed on shaking and clouded on heating, after adding a drop of 25 per cent nitric acid. The chicken was bled from the neck into small dishes, care being taken to keep the blood under as sterile conditions as possible. The blood was allowed to stand for a few hours until a clear serum separated which could be drawn off into a sterile pipette. The tests were carried out as follows: to 2 cc. of the protein solution in test-tubes were added two drops of the serum to be tested. The tubes were then incubated and observed at short intervals. In no case was there a positive precipitin test obtained. After a longer incubation a cloudiness was usually observed in the tubes, but the same occurred in the control when the serum was added to a salt solution alone. The addition of large amounts of the serum also caused a cloudiness in both the protein solutions and the control. As stated, all the results were negative. To check the method a white cockerel was sensitized against milk proteins by injecting 5 cc. of milk into the large vein under the right wing. 4 days later 10 cc. of milk were injected into the vein under the left wing. 6 days later 10 cc. of milk were injected intraperitoneally. After a further period of 10 days the cockerel was bled and positive precipitin tests were obtained when the serum was added to milk diluted 1 to 50 and 1 to 500. The fact that the precipitin tests were negative does not exclude the probability that leg weakness is due to an undue absorption of toxic substances and that the real function of roughage is to help in their ready elimination.

The fact that fowls suffering from leg weakness have an anemic appearance led us to make a number of hemoglobin examinations in the case of leg weakness as well as of normal specimens. The determinations were made using the Fleichl-Miescher hemometer. The chickens were bled from the wing vein. Determinations on six separate individuals suffering from leg weakness at various stages of severity showed a hemoglobin content varying from

7.45 per cent to 11.2 per cent. The amount found in normal birds averages 7.49 per cent. These data would indicate that in this disease there was not a subnormal hemoglobin content in the blood.

SUMMARY.

1. Our work points to the conclusion, which is in confirmation of the work of Osborne and Mendel, that the disease known among poultrymen as leg weakness is caused by the lack of a suitable roughage in the ration.

2. Among the roughages tried with our synthetic ration were paper, charcoal, dirt, agar, and fullers' earth. Paper in a concentration of 10 per cent of the ration was the most effective roughage used; agar was much less effective and fullers' earth almost worse than nothing.

3. The use of green cabbage, green clover, or of orange juice did not, in the absence of a suitable roughage, prevent the progress of this disease. Consequently we do not believe that leg weakness is a scurvy of chickens.

4. Provision for plenty of exercise or green feed will not prevent leg weakness if the bird at the same time is overfed a concentrated ration.

5. We obtained no evidence in cases of leg weakness that animals were sensitized to the proteins of their food. The blood did not show the precipitin reaction for the food proteins used. This would indicate that the food proteins as such were not traversing the intestinal wall. This negative evidence does not preclude the possibility of undue absorption into the blood stream of intestinal waste products in cases of leg weakness. The hemoglobin content of the blood of leg-weak chickens was not different from that of normal birds.

6. Our success with paper and the synthetic diet will make possible a study of the quantitative relations of the factors of nutrition in the growth of chickens.

EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. A typical case of leg weakness. This result was produced by our synthetic diet containing no roughage. Photographed when 90 days old.

FIG. 2. Reared on a synthetic diet containing 10 per cent of dirt. Weighed 1,092 gm. when photographed at 14 weeks of age. Although not fully feathered this bird appeared normal.

PLATE 2.

FIG. 3. Reared on a synthetic ration containing 20 per cent of charcoal. Weighed 994 gm. when photographed at 14 weeks of age. Although not fully feathered this bird appeared normal.

FIG. 4. Reared on a synthetic diet containing 10 per cent of paper reground with the ration. Weighed 710 gm. at 16 weeks and apparently was in perfect health.

FIG. 1.

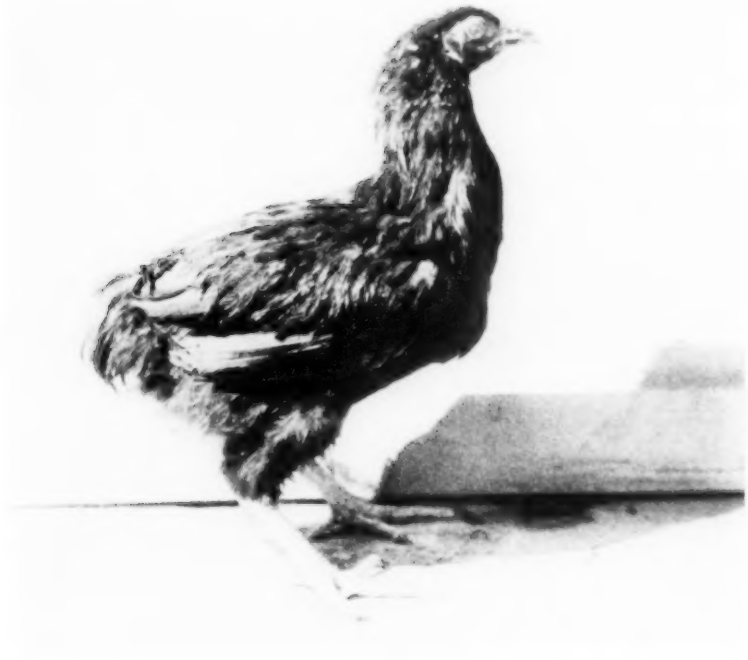

FIG. 2.

(Hart, Halpin, and Steenbock: Synthetic diets for baby chicks.)

FIG. 3.

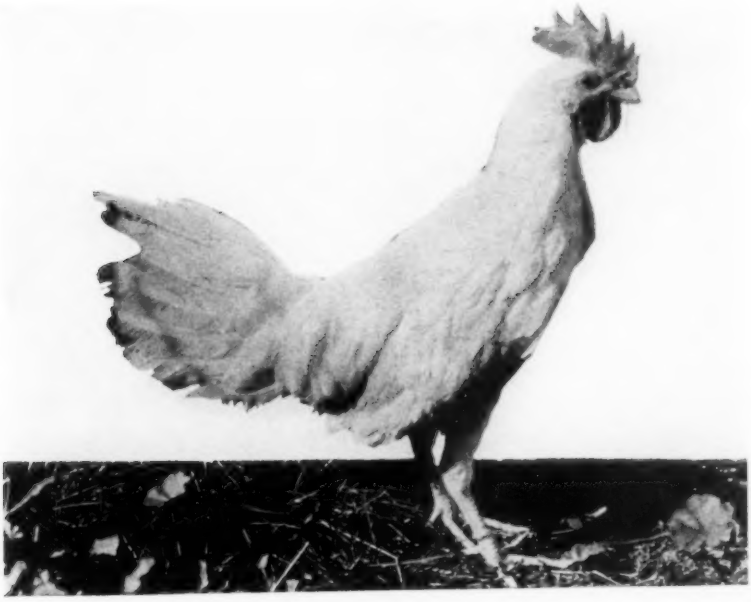

FIG. 4.

(Hart, Halpin, and Steenbock: Synthetic diets for baby chicks.)

AMINO-ACIDS IN NUTRITION.*

I. STUDIES ON PROLINE: IS PROLINE A GROWTH-LIMITING FACTOR IN ARACHIN (GLOBULIN FROM THE PEANUT)?

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Perhaps there is no problem in the realm of physiological chemistry that is more significant than the question of the synthetic power of the animal organism for the individual amino-acids of the proteins. That the animal body is able to synthesize glycine is a well established fact (1). That it requires tryptophane, the indole nucleus of the protein chain, and is unable to make it itself has been established by Willock and Hopkins (2), Abderhalden (3), Osborne and Mendel (4), and Wheeler (5). That the same is true of cystine is evident from the fact that Osborne and Mendel (6) procure such remarkable increases in weight of their animals when small amounts of isolated cystine are added to low planes of intake of casein, which has a low cystine content. Similar results were reported recently by Johns and Finks (7) in the case of phaseolin, the globulin and the main protein from navy bean.

In connection with lysine, Hart, Nelson, and Pitz (8), contrary to the findings of Osborne and Mendel (9), recently produced evidence that the mammary gland is unable to synthesize this complex.

With regard to tyrosine, we have conflicting evidence. Abderhalden (3) claims that this complex is essential for growth, while Totani (10) believes that the animal cell has the power of synthesizing the hydroxy-phenyl nucleus of the protein molecule.

* This article forms part of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University of Wisconsin.

Hopkins (11) has recently reported that arginine and histidine can replace each other when equimolecular amounts of either are added to the ration; also that with a diet made up of histidine, cystine, tyrosine, tryptophane, and lysine, that is a diet containing five out of the eighteen amino-acids of a protein, there occurs a remarkably slow loss of weight and long maintenance with apparent health. However, with a diet made up of leucine, valine, alanine, glycine, and glutamic acid, loss of weight is rapid and the animals soon succumb. Hopkins' work shows the relative indispensability of the ring compounds and the lesser importance of the monoamino-acids in proteins for nutrition.

EXPERIMENTAL.

As noted in this short review of the literature, no attempt has yet been made to determine the synthetic capacity of the animal cell for the pyrrole nucleus as found in proline. It was this problem which was submitted to experimental inquiry and the results thus far secured are presented in the following protocols and discussions.

As proline is present in considerable amounts in most proteins, it was not found possible to feed a protein entirely deficient in it, with the aim of ultimately making proline additions and noting the resultant improvement in the growth of the animals. Furthermore, as proline is not destroyed by hydrolysis or quantitatively precipitable, it was impossible to feed cleaved proteins from which all proline had been removed. Recourse therefore was had to the use of such proteins as have been reported low in proline, with the hope that their proline content might be sufficiently low to bring out possible limitations of the synthetic capacity of the animal. Three proteins which qualified with respect to a low proline content are edestin, lactalbumin, and arachin. In edestin, the globulin from hemp seed, about 1.7 per cent of the nitrogen is in the form of non-basic non-amino nitrogen (12), which is equivalent to 2.6 per cent of the protein as proline. In lactalbumin, the heat-coagulable protein from milk, Osborne and associates found 1.8 per cent of the nitrogen in the non-basic non-amino form (12), which presumably represents proline and oxyproline nitrogen. In the same protein

Crowther and Raistrick report 2.0 per cent of the nitrogen in the same fraction, using the same method of analysis (13). In the following experiments edestin was not used, as the large quantities necessary for the experiments are prepared only with difficulty. Lactalbumin also was not used as a primary constituent of the rations, because it was later found, as brought out in the following paper (14), that in this protein cystine and tyrosine are the growth-limiting amino-acids. Arachin alone, as isolated by Johns and Jones (15), was finally chosen as it is readily prepared and is the lowest in proline of any protein yet reported. Their analysis of this protein follows (16):

	per cent
Glycine	0.00
Alanine	4.11
Valine	1.13
Leucine	3.88
Proline	*1.37*
Phenylalanine	2.60
Aspartic acid	5.25
Glutamic acid	16.69
Serine	—
Oxyproline	—
Tyrosine	5.50
Cystine	0.85
Arginine	13.51
Histidine	1.88
Lysine	4.98
Tryptophane	Present.
Ammonia	2.03
	63.78

It will be noted from the above table that arachin is a complete protein in so far as it contains all the amino-acids, with the exception of glycine and possibly serine and oxyproline, thus far isolated as final hydrolysis products of proteins by chemical methods. It is rather low in cystine and in proline. It is also rather low in histidine, containing only 1.88 per cent, but remarkably high in arginine, 13.51 per cent, and, if Hopkins' idea of the transformation of arginine and histidine into each other by the animal cell is correct, then there ought to be more than sufficient arginine to take care of the possible deficiency of histidine. Tryp-

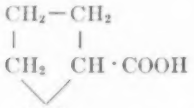
tophane evidently was not determined quantitatively and the data are only qualitative. In order to eliminate cystine and tryptophane as possible limiting factors before a response to proline addition could be expected, they were added to the extent of 1.0 and 0.5 per cent of the protein, respectively. Since casein is reported by Hopkins and Cole (17) to contain only 1.5 per cent tryptophane, and, furthermore, since arachin is shown to contain this amino-acid, 0.5 per cent additions of tryptophane were considered to be sufficient to eliminate that constituent as a possible limiting factor.

All the data that were collected were obtained using rats as the experimental animals. The rats were confined in wire cages in small groups and kept under sanitary conditions, being at all times abundantly provided with the rations fed and with distilled water. Among the ingredients in the ration, the water-soluble vitamine was introduced in the form of an alcoholic extract of wheat embryo—previously extracted with ether—in such amounts that each 100 gm. of ration carried the extract from 15 gm. of wheat germ. The extract was evaporated on the dextrin before being introduced in the ration. The salt mixture was one previously used in this laboratory (18).

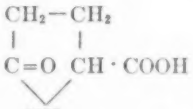
The proline used in this feeding work was first prepared by the Fischer ester method (19). Proline has no amino group and consequently should contain no amino nitrogen; however, both the racemic and optically active proline contained 10 to 12 per cent amino nitrogen. This foreign amino nitrogen could come only from alanine, leucine, and valine, or the leucine fraction; and, therefore, the latter was fed as a control. During the process of distillation of the esters it was not always possible to maintain as low a pressure as 0.5 mm. and at times 2 to 3 mm. pressures were employed and, therefore, the temperature often had to be raised slightly, possibly carrying over traces of the next fraction of esters. Such a condition may very well have influenced, at least partly, the solubility of some amino nitrogen in the presence of proline in absolute alcohol. Later, proline was prepared by the method of Fischer and Boehner (20) not involving esterification. By slightly modifying the technique of these authors by introducing phosphotungstic acid to precipitate the bases, proline was obtained which contained only 5 per cent

amino nitrogen. The fact that Van Slyke (21) introduced a method for determining proline by the difference between the total and amino nitrogen of the alcoholic extracts of the leucine fraction in itself suggests that it is not always possible to procure proline absolutely free from some amino nitrogen.

Supplementary to the experiments where proline was added to the ration some attempts were made to use pyrrolidone carboxylic acid instead. The great similarity in structure of these two compounds



Proline



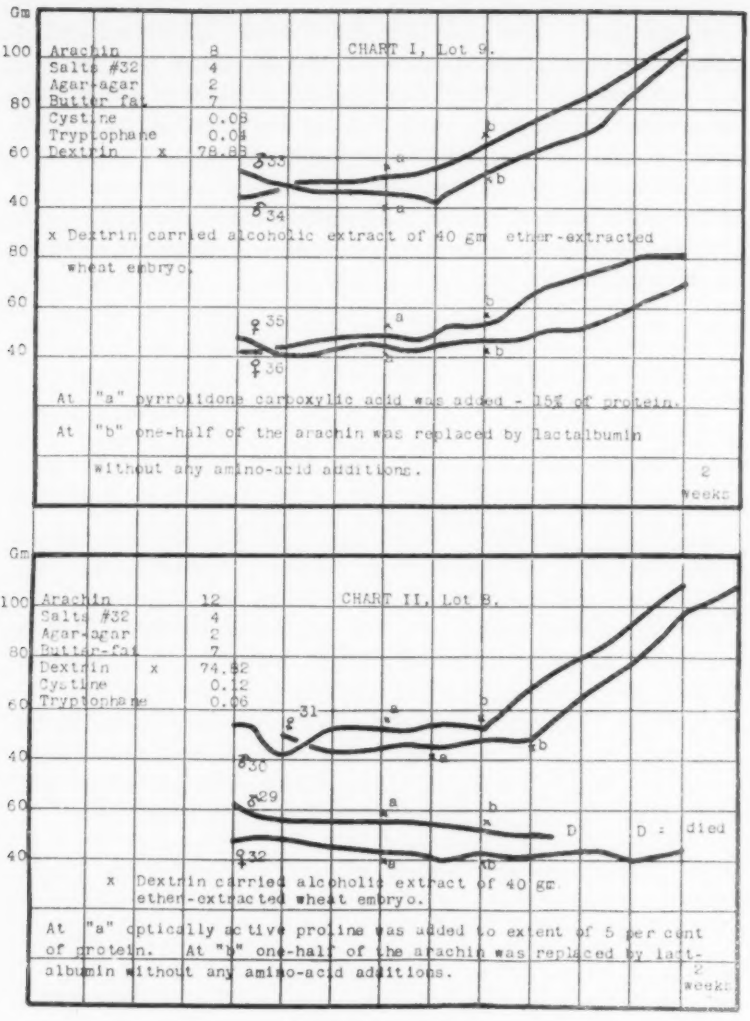
Pyrrolidone carboxylic acid

and the ease with which pyrrolidone carboxylic acid is formed from glutamic acid made a determination of the possible functions of this derivative very interesting. It was prepared in quantity by Foreman's method (22) and was used in a few experiments.

With the aim of ascertaining the conditions under which supplementary additions of proline might be expected to produce the most decisive results, it was necessary to determine first the increments of growth possible on various planes of arachin intake. As no data on the biological efficiency of arachin were available, it was necessary to establish these fundamental relations which are brought out in the following protocols.

Chart I, Lot 9.—Arachin obviously gives no indication of containing a mixture of amino-acids of unusual value for satisfying the requirements of the growing organism, otherwise an intake at a level of 8 per cent of the ration—especially when supplemented with cystine and tryptophane—should have made possible more than maintenance in these young rats. In this lot in a preliminary test at "a" a 0.12 per cent addition of pyrrolidone carboxylic acid was made with negative results, but when at "b" 4 parts of arachin were replaced by lactalbumin—also in itself a poor protein—some growth was made possible.

Chart II, Lot 8.—12 per cent of arachin supplemented with cystine and tryptophane gave no indication of possessing greater value for inducing growth in rats on an otherwise satisfactory ration than 8 per cent of arachin (Chart I, Lot 9). When at "a" there was added in addition to the cystine and tryptophane l-proline equivalent by weight to 5 per



CHARTS I AND II.[1]

cent of the arachin no improvement resulted, but when later one-half of the arachin was replaced by lactalbumin, as in Chart I, growth was made possible.

Chart III, Lot 15.—Even when fed at an 18 per cent level and when supplemented with cystine and tryptophane, arachin proved to be a poor protein. A small amount of growth resulted at this intake but it was not

[1] In all charts, for "40 gm. ether-extracted wheat embryo" read "15 gm. ether-extracted wheat embryo."

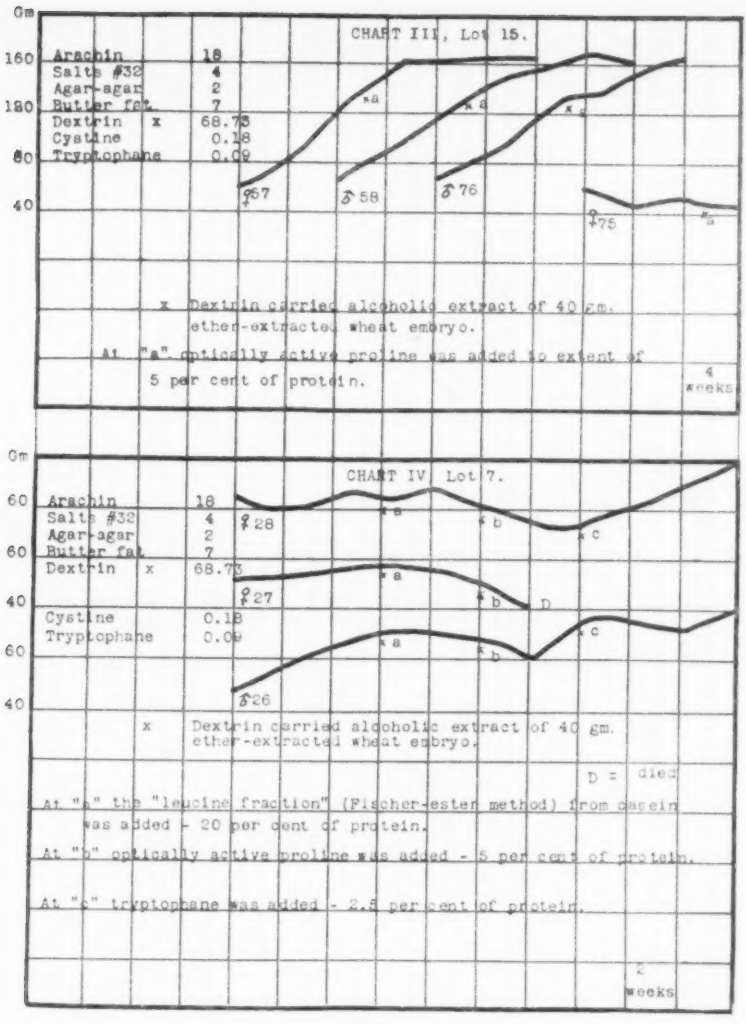
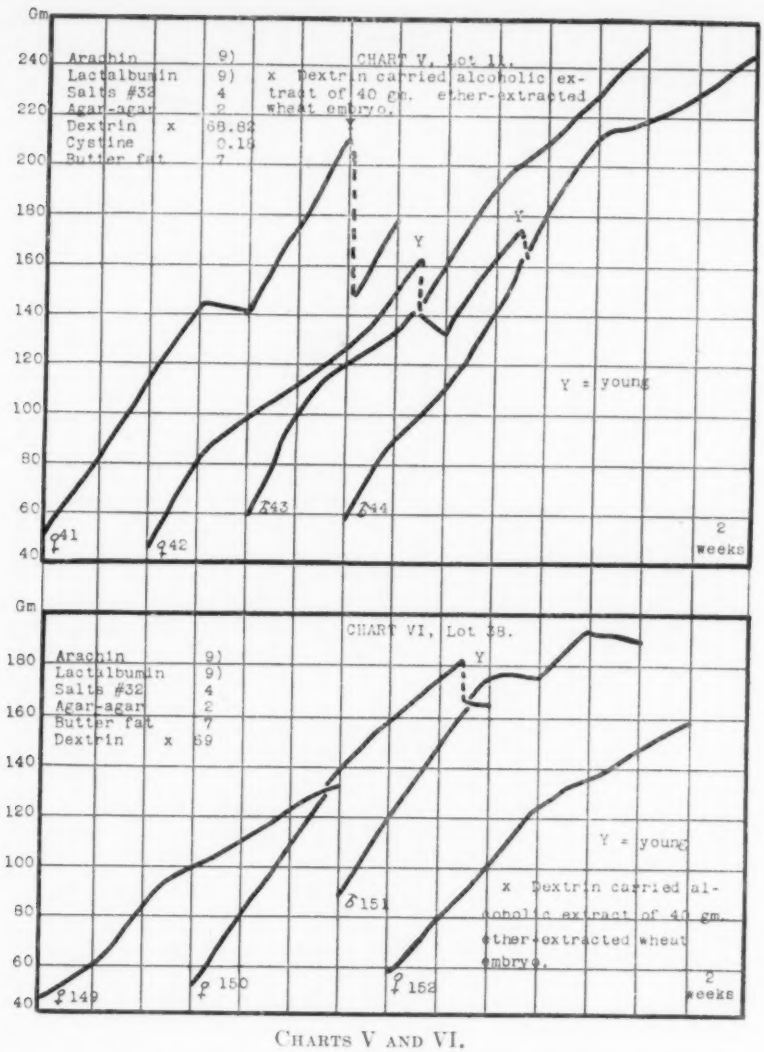


CHART III, Lot 15.
Arachin 18
Salts #32 4
Agar-agar 2
Butter fat 7
Dextrin x 68.73
Cystine 0.18
Tryptophane 0.09
♀67
♂58
♂76
♀75
4 weeks
x Dextrin carried alcoholic extract of 40 gm. ether-extracted wheat embryo.
At "a" optically active proline was added to extent of 5 per cent of protein.
CHART IV Lot 7.
Arachin 18
Salts #32 4
Agar-agar 2
Butter fat 7
Dextrin x 68.73
Cystine 0.18
Tryptophane 0.09
♀28
♀27
♂26
D
2 weeks
x Dextrin carried alcoholic extract of 40 gm. ether-extracted wheat embryo.
D = died
At "a" the "leucine fraction" (Fischer-ester method) from casein was added - 20 per cent of protein.
At "b" optically active proline was added - 5 per cent of protein.
At "c" tryptophane was added - 2.5 per cent of protein.

CHARTS III AND IV.

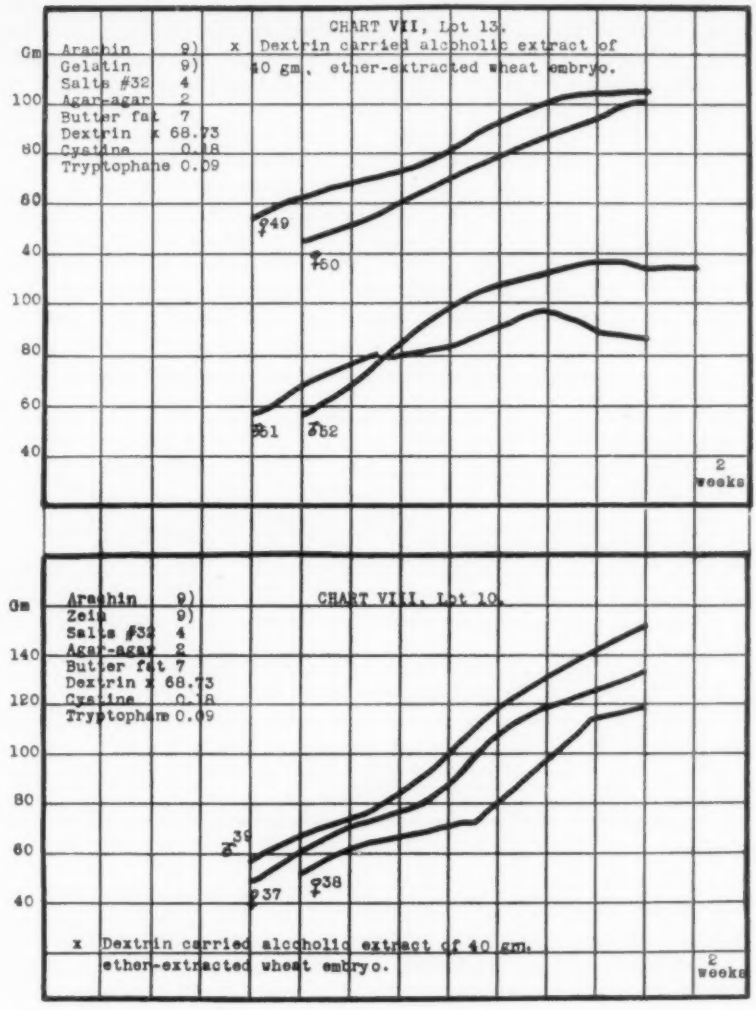
maintained even when at "a" l-proline to the extent of 0.9 per cent of the ration or 5 per cent of the protein was added.

Chart IV, Lot 7.—Here the feeding of the basal ration containing 18 per cent arachin as in Chart III, Lot 15, was duplicated with, however, less resultant growth and this in spite of the fact that adequate consumption



CHARTS V AND VI.

for growth was obtained. At "a" the ration was supplemented with a leucine fraction (Fischer ester distillation) from casein, and at "b" by l-proline in addition. Though the proline was added in an amount equivalent to 5 per cent of the arachin, and at "c" an additional amount of tryptophane was introduced, so that in all 2.5 per cent of the weight of protein in tryptophane had been added, no growth resulted. Alanine,



CHARTS VII AND VIII.

leucine, valine, cystine, tryptophane, and proline are, therefore, eliminated as primary factors in the limitation of growth of the animal when arachin is the sole source of protein.

Chart V, Lot 11.—Of the probable supplementary value of lactalbumin and arachin some evidence has already been presented (Charts I and II). Here we have indications that in the presence of cystine the supplementary effect is of a high order of efficiency.

Chart VI, Lot 38.—In the absence of additional cystine very good supplementary effect was produced. On the whole, the performance of the animals was not so good here as in Chart V, Lot 11, but this may be considered eliminated when consideration is given to the fact that this lot contained three females instead of two.

Chart VII, Lot 13.—Subjecting the failure of l-proline to enhance efficiently the value of arachin for production of growth in the rat to further analysis, it is seen that gelatin, though it is very rich in proline—7 per cent—likewise is of little or no value. Conditions were, however, somewhat different from those obtained with the rats on the arachin-cystine-tryptophane diets, as these animals were very irritable, became emaciated, and lost some of their hair. In spite of this, somewhat more growth was obtained, yet the increment was not sufficient to be of decisive value.

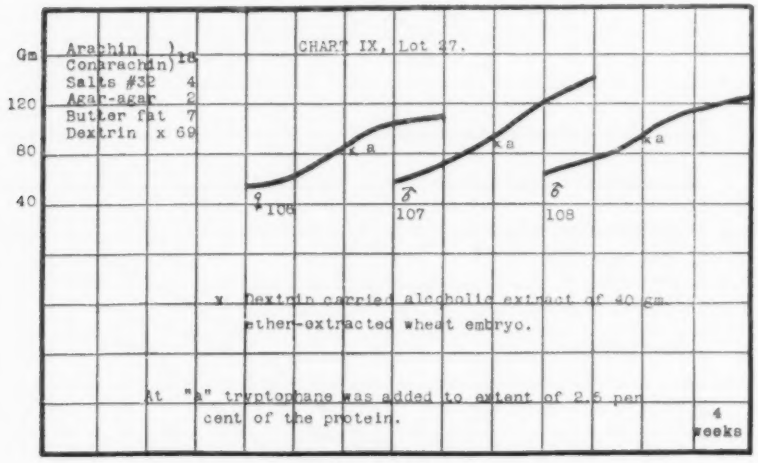


CHART IX.

Chart VIII, Lot 10.—Zein, another protein high in its proline content—9 per cent—when used as a supplement to arachin brought the animals into the same state as gelatin, with this exception that after 8 weeks there ensued recovery from their poor nutritive condition; yet little growth resulted. It was surmised that failure of growth might have been due to the indigestibility of the zein, but an attempt to feed hydrolyzed zein led to failure as the animals refused the ration.

Chart IX, Lot 27.—While not strictly germane to the subject under consideration, yet it was of interest to determine the biological value of the mixture of globulins, arachin and conarachin, as they are extracted from the peanut by salt solutions. As seen on the chart, no better results were obtained with these than with arachin alone. Tryptophane added at "a" to the extent of 2.5 per cent of the protein proved not to be the limiting factor.

DISCUSSION.

In the data presented it is shown that arachin is biologically a poor protein. Other proteins showing a similar distribution of amino-acids have been found to be good proteins. It is, however, true that the available analysis of arachin, as with other proteins, leaves much to be desired, because only a fraction, 63 per cent, of the amino-acids has been isolated (16). Assuming that arachin was either a good or poor protein in spite of or by virtue of its low proline content, 1.37 per cent, proline additions were made. When these additions were made, even in the presence of additional cystine and tryptophane, which have already been found to be growth-limiting factors with other proteins, negative results were obtained. Furthermore, the fact that neither gelatin nor zein, which are both high in proline, supplement this globulin of the peanut gives evidence that proline is not the growth-limiting factor in arachin.

With lactalbumin efficient supplementation was secured but unfortunately this does not give unequivocal evidence as to the character of the deficiency of arachin. It cannot have been tryptophane which is contained in lactalbumin, because in one experiment this amino-acid was added to the extent of 2.5 per cent of the arachin (Chart IV, Lot 7; Chart IX, Lot 27) and no response was secured. It also cannot have been cystine because cystine added equivalent to 1 per cent of the arachin, in the presence of tryptophane, was also ineffective and furthermore lactalbumin itself is deficient in this amino-acid (14).

It is also true that arachin is rather low in histidine, 1.88 per cent, but on the whole, judging from its chemical composition, and also making additional allowance for the 37 per cent of the amino-acids that were lost in manipulation, this protein ought to be fairly good for growth. Actually, however, even with additions of cystine, tryptophane, proline, alanine, leucine, and valine, it serves for little better than maintenance.

Although according to present data arachin contains in quantity all the amino-acids believed to be necessary for the construction of mammalian body tissue, it was in these experiments found inadequate for maintaining growth in the rat. For this apparent inconsistency between chemical and biological tests

there is no explanation at present. It is quite possible, however, that this anomalous condition may be clarified if we accept that constitutional differences as well as qualitative and quantitative distribution of amino-acids may determine the biological value of a protein. It is quite possible that the chemical structure of the protein may be such that after digestion certain of the simple peptides containing some amino-acid essential for the construction of body tissue escape further cleavage and present themselves in a form unacceptable for assimilation into body tissue. In this connection it is to be remembered that, while amino-acids have been isolated from blood and while an increase in non-protein, non-urea nitrogen and an increase in free amino nitrogen has been observed during digestion, it is still not evident as to the form in which the nitrogen is assimilated by the tissues from the blood stream. It may be absorbed as peptides or as amino-acids or as both. In the latter case the quantitative relations between these processes would determine the issue. It is not intended to enter into a discussion of this phase of the subject further than to point out how imperfect hydrolysis may modify assimilatory processes. For differences in the cleavage of peptides by enzymes the work of Abderhalden done *in vitro* gives abundant evidence.

Fischer and Abderhalden (23) demonstrated that *in vitro* trypsin is able to hydrolyze certain polypeptides while it will not split others due to differences in arrangement. To give the simplest example, alanyl-glycine, $CH_3.CHN_2.CO.NH.CH_2COOH$, is hydrolyzed while its isomer, glycyl-alanine, $NH_2.CH_2.CONH.CH.(CH_3)COOH$, is not. Numerous more complicated instances are cited but it is sufficient to say that configuration is an important factor determining whether certain polypeptides will be and whether others will not be hydrolyzed by enzymes. That a similar situation may exist in the digestive tract of the animal organism is suggested; and, until further experimental data based on specific amino-acid additions are obtained which may improve the poor quality of arachin, it is tentatively proposed that the deficient character of arachin is to be attributed to its stereochemical rather than to its chemical composition.

It remains, however, to establish that proteins differ in the arrangement of their component amino-acid constituents to the extent that incomplete hydrolysis in a given time would obtain with one and not with the other. As far as known to the author differences in the digestibility or rate of digestion of peptides isolated from proteins has not been demonstrated.

Although arachin is deficient as a food protein, it is interesting to note that Daniels and Loughlin (24) have recently shown that the total proteins of the peanut are satisfactory for growth. Peanuts, then, must have other proteins that supplement arachin.

SUMMARY.

1. Arachin is biologically a poor protein in spite of the fact that chemical analysis indicates absence of no necessary amino-acid.

2. *Proline is not a growth-limiting factor in arachin.*

3. Neither gelatin nor zein, which are high in proline, supplements arachin. This fact is additional evidence that proline is not the nucleus responsible for the poor biological value of that globulin of the peanut.

4. Tryptophane and cystine when added to the extent of 2.5 and 1.0 per cent of the total protein respectively did not improve the deficient character of arachin.

5. The leucine fraction, composed of a mixture of alanine, leucine, and valine, did not improve the nutritive value of arachin.

6. Lactalbumin, in itself a deficient protein biologically, supplements arachin, but to a much greater extent in the presence of cystine.

7. Arachin is not supplemented by its sister globulin, conarachin.

The author wishes to express his appreciation to Professor E. B. Hart and Professor H. Steenbock for many valuable suggestions and criticisms in connection with this work.

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AMINO-ACIDS IN NUTRITION.*

II. THE NUTRITIVE VALUE OF LACTALBUMIN: CYSTINE AND TYROSINE AS GROWTH-LIMITING FACTORS IN THAT PROTEIN.

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Since 1911, when Osborne and Mendel published their first work on "Feeding experiments with isolated food substances," there has been made accessible to investigation the problem of the biological evaluation of proteins as correlated with their chemical analyses. It is true that for a time the advance made in the solution of this problem was obscured by the lack of appreciation of the necessity of the presence of the water-soluble and fat-soluble vitamines, but this was soon rectified as information in connection with these vitamines was accumulated by Hopkins (1), McCollum and Davis (2), and by Osborne and Mendel themselves (3). However, with the incorporation of the water-soluble vitamine in the diet in the form of the extracts in which it is known new complications were introduced, inasmuch as this also meant the introduction of a small amount of nitrogen in unknown forms. Some of the earlier work in this field was, therefore, open to much criticism, as unquestionably in many instances the amino-acid deficiency of a protein was covered by the amino-acid nitrogen contained in the vitamine preparations used in excessive amounts. Even with the present technique of ration-compounding this difficulty has not been entirely circumvented, but it has at least been minimized by the use of vitamine preparations low in nitrogen and minimum in amount (4).

* This article forms part of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University of Wisconsin.

In the early work of Osborne and Mendel, lactalbumin no more than edestin or other globulins was able to support growth, but here the fat-soluble vitamine was not incorporated in the diet and little else was to be expected. In 1916, in a series of experiments designed to test the comparative values of casein, lactalbumin, and edestin, these same workers found that lactalbumin was a protein of unusual value; in fact, 50 per cent more casein and 90 per cent more edestin than lactalbumin were required to produce the same increment in growth (5). Later, however, in another series of experiments they found lactalbumin, as well as cotton-seed globulin, cotton-seed protein, or squash seed globulin, invariably unable to support growth (6). These conflicting data were harmonized when it was appreciated by them that where failure had supervened yeast had been used as the source of the water-soluble vitamine, while where success had been obtained their so called protein-free milk had been used instead. Evidently, then, here occurred a concrete instance of supplementation of an "incomplete" protein by the nitrogen of a water-soluble vitamine preparation from milk. Possibly the statement of McCollum, Simmonds, and Parsons (7) that lactalbumin is a poor protein can also be harmonized on these general premises. Another suggestion has been made by Emmett and Luros (8), in which they state that lactalbumin is a complete protein and that their success was due to a vitamine, with a possible detoxifying effect, carried by the lactose and not introduced by other investigators. It scarcely appears necessary, however, in the light of present information to advance such hypotheses, in addition to those already advanced, which are based on the supplementary value of the nitrogen carried by the vitamine preparations. It becomes increasingly desirable, however, to establish definitely the specific amino-acids which limit the nutritive value of lactalbumin.

EXPERIMENTAL.

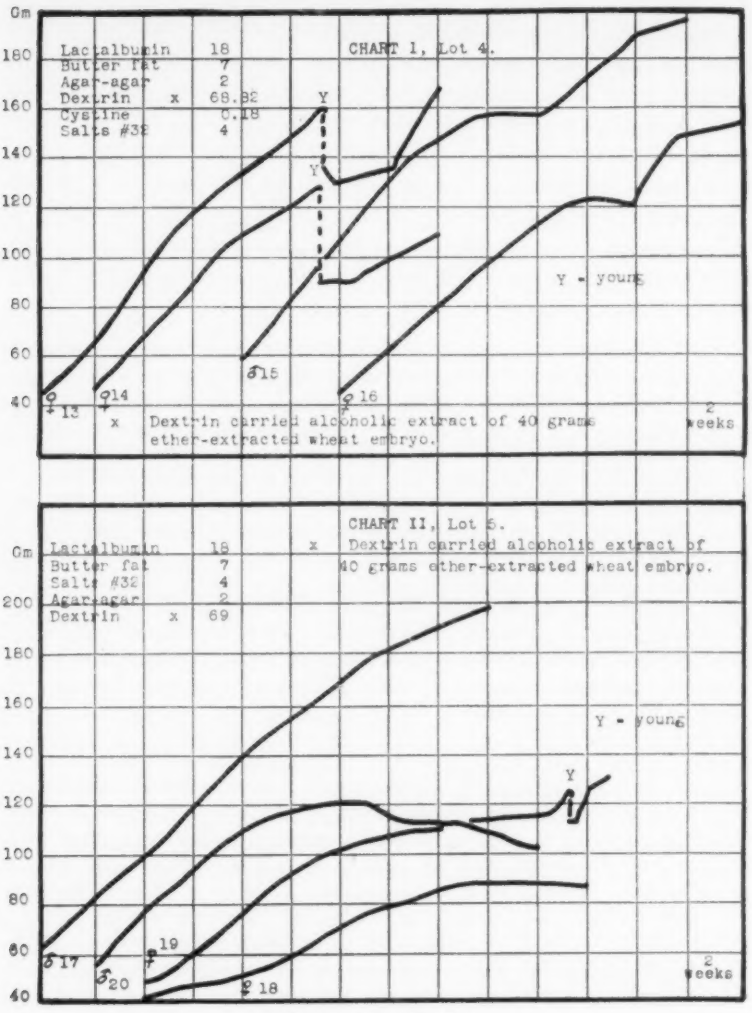
All the experiments were carried out on young rats according to the technique which has become a standard in this laboratory in the course of the last few years. As basal constituents there were used dextrinized corn-starch, agar-agar for roughage, Salt No. 32 for mineral constituents, filtered butter fat for fat-soluble

vitamine, and an alcoholic extract of ether-extracted wheat embryo for water-soluble vitamine. Before the experimental details were fully organized one ration containing yeast as the source of water-soluble vitamine was started, but this was later changed as the wheat embryo preparation was found to introduce only one-third as much nitrogen as the yeast. Although comparative supplementary values could not be forecast, it appeared to be desirable on general premises to introduce the minimum of nitrogen that might be of possible supplementary value.

As the individual proteins of milk may not be as definite entities as is ordinarily assumed, it appears desirable to describe the method of preparation of the lactalbumin used in some detail. The small amount of so called albumin in milk as obtained by heat coagulation after the removal of the casein made it almost impossible to consider milk as a source for its preparation. Instead there was used the whey produced in the manufacture of cottage cheese by the lactic acid fermentation process. 1,000 to 2,000 pound lots of this whey were run through a centrifugal whey separator to remove small particles of casein held in suspension and then heated by steam to about 85°C. for 1 hour. Care was taken to avoid violent agitation, as otherwise the coagulated albumin particles do not coalesce and their collection is made difficult. After settling for 1 hour the supernatant liquors were run off through a large wire strainer covered with three layers of cheese-cloth. The albumin left in the vat was united with that caught by the strainer and after standing for 24 hours was decanted free from excess liquors and worked up repeatedly by hand with 40 liters of chloroformed tap water for a week. This was done to wash out soluble forms of nitrogen of possible supplementary nutritive value. The supernatant liquor was finally allowed to drain off through the strainer, and the albumin dried in a steam oven at a temperature not exceeding 75°C. Final purification with hot 95 per cent alcohol left a product faintly tinged with yellow. On analysis it was found to contain from 0.5 to 0.7 per cent ash, 0.06 per cent phosphorus, and 14.3 to 14.7 per cent nitrogen.

Chart I, Lot 4.—Lactalbumin fed at an 18 per cent level in a low caloric ration and when supplemented with cystine to the extent of 1 per cent of the weight of the protein met the requirements for growth in the rat.

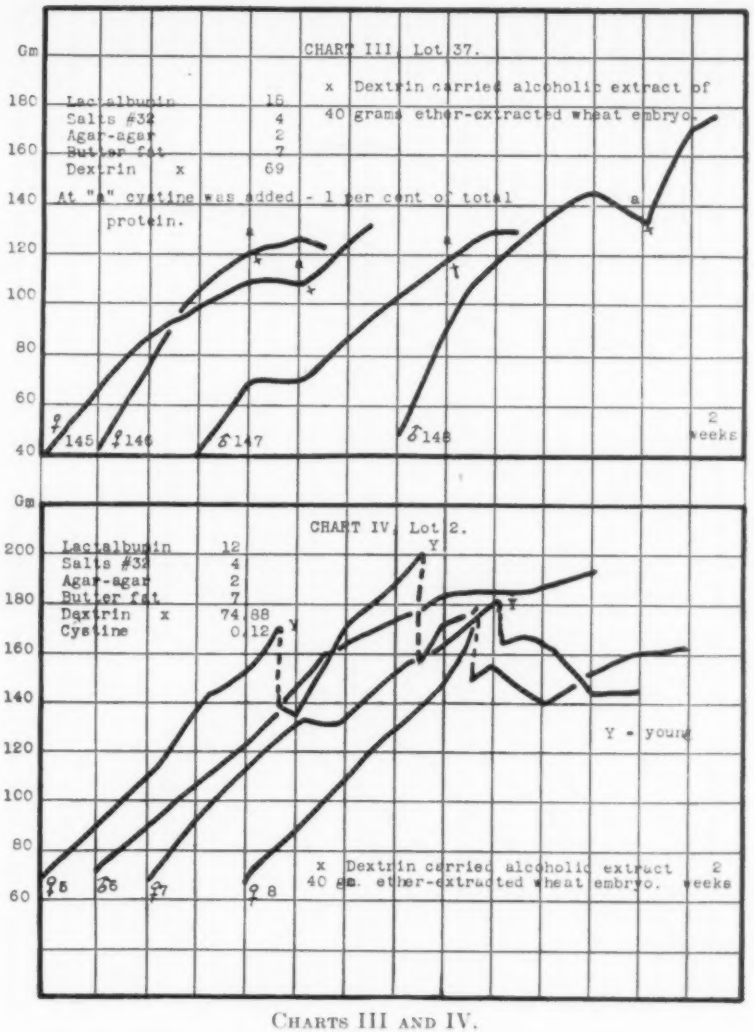
Chart II, Lot 5.—By comparison with Chart I, Lot 4, it is seen that when the cystine is omitted from the ration the growth of the animals is far from being satisfactory. Of the four animals fed on this ration one was able to grow at a rate which may be considered normal, but, on the other hand, three failed to continue their growth. This is to be taken as indicating a deficiency of the ration, for while the one animal was able to grow, that ability no doubt was resident in either a larger consumption of the ration, or else in a greater efficiency in conserving available sulfur complexes in the body.



CHARTS I AND II.[1]

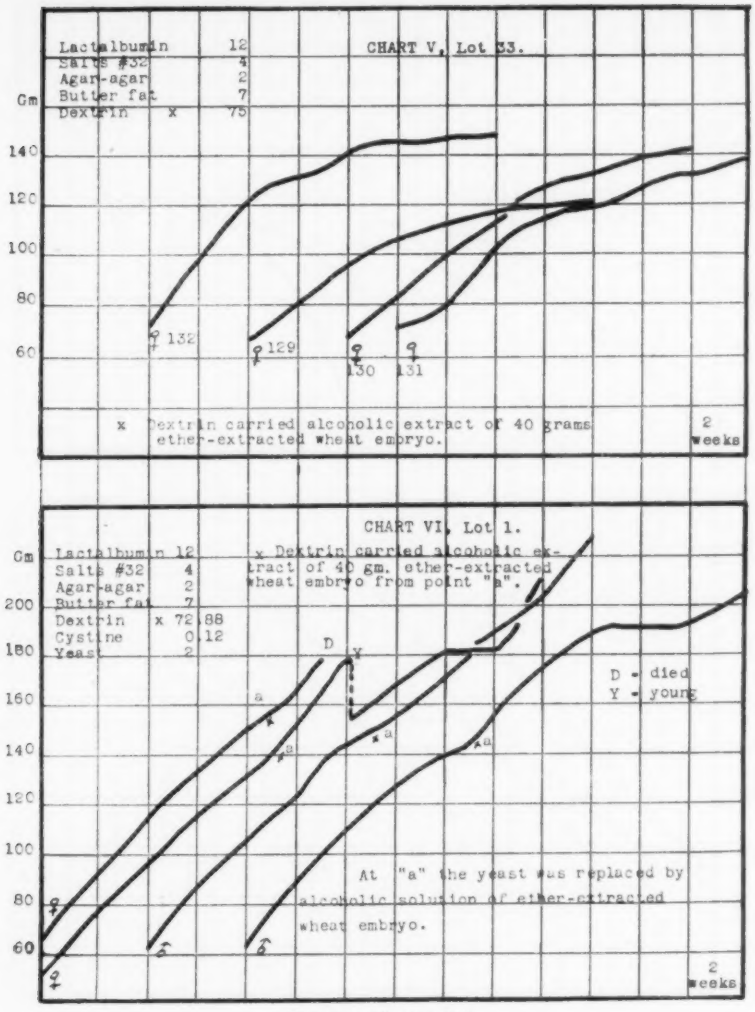
Chart III, Lot 37.—A duplication of the experiment of Chart II, Lot 5, resulted in good growth of the four animals for a considerable period, but ultimately failure ensued in two animals out of the four in the time that the trial was continued. When at this point the ration was fortified with cystine there resulted prompt recovery. This emphasizes the point indi-

[1] In all charts, for "40 gm. ether-extracted wheat embryo" read "15 gm. ether-extracted wheat embryo."



CHARTS III AND IV.

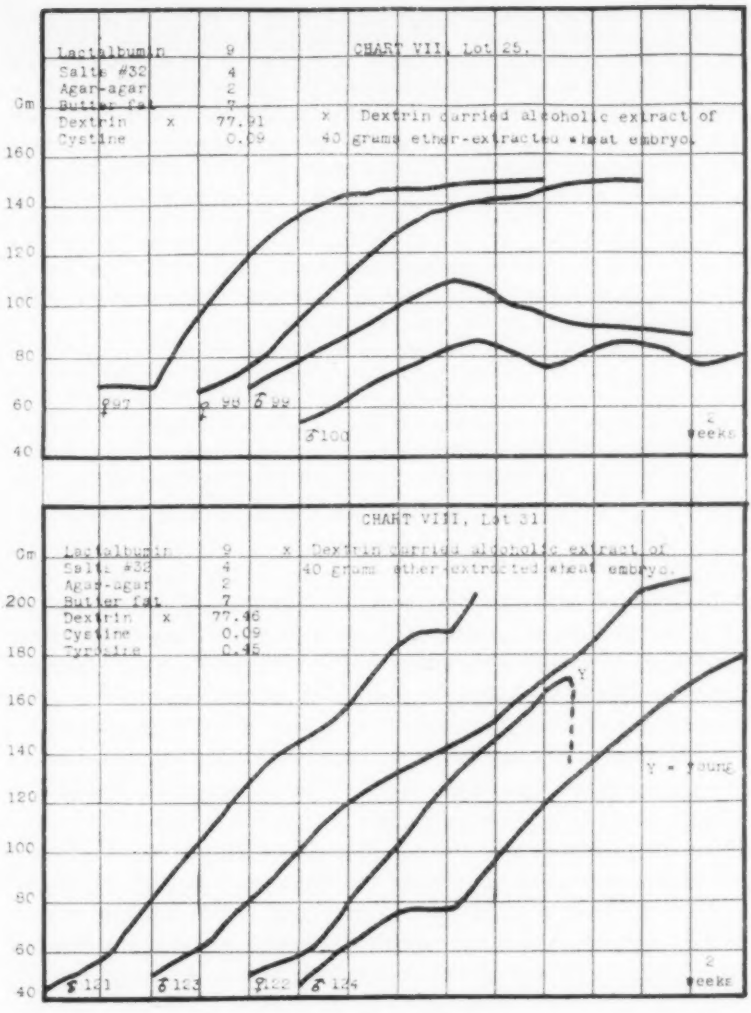
cated in the previous chart that cystine is a limiting factor in the biological efficiency of lactalbumin. The fact that the two animals did not respond to the addition of cystine does not detract from the value of the data, as this may well have been due to irreparable injury directly or indirectly caused by the amino-acid deficiency.



CHARTS V AND VI.

Chart IV, Lot 2.—Lactalbumin at a 12 per cent level supplemented with 1 per cent cystine supported a fair amount of growth.

Chart V, Lot 33.—When not supplemented with cystine at a 12 per cent lactalbumin level the growth performance was considerably inferior (Chart IV, Lot 2).



CHARTS VII AND VIII.

Chart VI, Lot 1.—When yeast was used as the source of the water-soluble vitamine, lactalbumin on a 12 per cent level supplemented with 1 per cent of cystine allowed the rats to make excellent growth. At "a" the yeast was replaced by an alcoholic extract of ether-extracted wheat embryo, such as was used in the other rations, and growth continued for the

duration of the trial. While in itself not conclusive, as the supplementary value of the yeast alone was not determined, yet Osborne and Mendel's findings that lactalbumin was a poor protein when yeast was used as a source of the water-soluble vitamine strongly suggest that the addition of cystine was here responsible for the excellent growth. The continuation of growth when the change in water-soluble vitamine from yeast to an alcoholic extract of wheat embryo was made bears out this suggestion.

Chart VII, Lot 25.—When supplemented with 1 per cent of cystine no such increment in growth is obtained with the lactalbumin fed at a 9 per cent level as when fed at a 18 or even 12 per cent level. At this low level of protein intake evidently other amino-acids are reduced in amount below the level where they can still allow growth to take place after the primary deficiency of cystine has been removed.

Chart VIII, Lot 31.—The amino-acid responsible for failure of growth in Chart VII, Lot 25, appears to be tyrosine. Growth was regular and long continued when the lactalbumin fed at a 9 per cent level was supplemented with tyrosine as well as cystine. From this it appears that when an alcoholic extract of wheat embryo is used as the source of the water-soluble vitamine tyrosine is the second growth-limiting factor in lactalbumin.

DISCUSSION.

It appears demonstrated that lactalbumin, if we accept as lactalbumin the proteins in milk removable by heat coagulation after the casein has been removed, is biologically an incomplete protein. This was brought out in feeding trials even when nitrogen in unknown forms had been introduced with the water-soluble vitamine in the ration, so that it constituted 2 per cent of the total protein nitrogen. Under the experimental conditions cystine proved to be an efficient supplement to the lactalbumin when the protein was fed at an 18 and at a 12 per cent level; yet lactalbumin is not unusually low in cystine, as it has been reported to have 1.30 per cent of its total nitrogen in the form of cystine or 1.74 per cent molecular cystine (9), which is about the average percentage of cystine in other proteins (10). Although arachin contains only about one-half as much cystine (0.88 per cent cystine nitrogen) as lactalbumin, no response to cystine additions was obtained in the case of arachin, even in the presence of tryptophane (11). This fact the author believes is further evidence to substantiate the theory set forth in a previous communication (11) that the efficiency of a protein may largely depend on its constitution as well as composition in amino-acids, and that

chemical analysis of amino-acid content may at times be inadequate to explain the nutritive failure or success obtained with certain proteins widely distributed in nature. It is quite possible that cystine is so oriented in the complex polypeptide chain of lactalbumin that when it is hydrolyzed by the enzymes in the digestive tract it is split into simpler peptides of such form that a great part of them escape further cleavage, and are, therefore, deaminized and converted to urea. Of course, it still leaves open the possibility that the animal organism requires more cystine than that actually contained in lactalbumin when that protein is fed at as high a plane of intake as 18 per cent of the total food.

Osborne and Mendel found lactalbumin to be a highly efficient protein when fed with protein-free milk, and the experimental data here presented show that cystine renders that protein of excellent nutritive value, when employing a synthetic salt mixture, and an alcoholic extract of wheat embryo for water-soluble vitamine, when without cystine it is of little value. The author is consequently of the opinion that protein-free milk must either carry cystine as part of its nitrogen of unknown source, or other forms of organically bound sulfur which the animal organism can readily transform into cystine. An analysis of protein-free milk showed a sulfur content of 0.2 per cent, of which the greater part was in organic combination.

While growth was induced by the addition of cystine to lactalbumin when fed at 12 and 18 per cent levels, such was not the case when the protein was reduced to a 9 per cent level. This brings out the fact that, while cystine is the primary growth-limiting factor in lactalbumin, other amino-acids may also limit its usefulness when the intake is sufficiently reduced. With the protein under consideration this proved to be tyrosine, as complete success was obtained when the lactalbumin was fortified with 5 per cent of its weight of tyrosine.

Prior to this work the experimental evidence does not give definite information with regard to the indispensability of tyrosine for growth. The first notable experiment on this subject was conducted by Abderhalden (12) on a dog. A preparation of the digestion products of casein, freed from tyrosine as completely as possible, was given to the animal. The dog lost 750 gm. in 9 days and there was a further loss of weight in

the 4 following days, though the loss of weight in the last few days may have been partly due to an insufficient intake of food. The loss of weight, however, was regained almost entirely when tyrosine was added to the previously consumed dietary. Abderhalden, therefore, came to the conclusion that tyrosine is an essential amino-acid for nutrition.

Recently Totani (13) continued the work on tyrosine with a view to the possible replacement of that amino-acid by its corresponding ketonic acid. Since Abderhalden fed his protein freed from tyrosine by crystallization only, Totani made a much more painstaking and laborious attempt towards removing all traces of tyrosine from a casein-hydrolysis mixture. He found that after repeated crystallizations the material still gave a positive Millon's test. Mercuric sulfate and phosphotungstic acid were then used to precipitate out any residual amounts of tyrosine. He then found that the most efficient method of exhausting casein of its tyrosine content was to use a combined tryptic and acid hydrolysis. With all his most elaborate methods his final preparation of hydrolyzed protein was found to contain by colorimetric measurements 0.3 per cent tyrosine. Totani found that animals which received tyrosine did not produce any more growth than those that were on the practically tyrosine-free ration, and concluded from these experiments that tyrosine is not essential for nutrition, thereby taking a stand opposite to Abderhalden.

Totani also found that, while tryptophane improves somewhat the deficient character of gelatin, surprisingly, tyrosine, which is not found in that protein, does not; also that the addition of phenylalanine produces no effect just like tyrosine. This strengthens his evidence, he believes, that tyrosine is an amino-acid unessential for growth and that phenylalanine can be transformed into hydroxy-phenylalanine or tyrosine.

There are several objections to Totani's work, the main being that his period of experimentation, 24 days, was altogether too short to get any idea of the possible outcome of such an investigation. Besides, as Totani admits himself: "It is, of course, an objection to my experiments that the actual amount of tyrosine left in the diet is uncertain." He claims he could detect only 0.3 per cent tyrosine, but that figure may be much higher and we do not know yet about the minimum requirements of that amino-acid for growth. Moreover, in his control ration his rats did not grow normally, and from the experience in this laboratory on the requirements of the water-soluble vitamine, Totani's alcoholic extracts of protein-free milk did not furnish enough of that factor in the diet. In his work on gelatin the records show loss of body weight in all cases. The addition of tyrosine brought no improvement, neither did the equivalent amount of phenylalanine, yet the investigator concludes that the latter may be transformed into the former.

The author believes that Totani's conclusions with regard to the possible synthesis of the benzene nucleus are unwarranted and are based on altogether inadequate data. On the contrary the experimental data here presented strongly suggest the indispensibility of tyrosine for growth. However, further experimental data are necessary to establish this point finally.

Reconsidering the supplementary value of protein-free milk, it is interesting to note that, although Osborne and Mendel claim they were unable to obtain a positive Millon's test on 2 or 3 gm. of preparations of protein-free milk (14) the author has repeatedly obtained positive tests for tyrosine in preparations of several tenths of a gm. of protein-free milk prepared exactly as described by Osborne and Mendel. The preparations contained only 0.60 to 0.65 per cent nitrogen, and, therefore, were certainly free from any notable amounts of casein or lactalbumin. When 28 per cent of the ration is made up of this protein-free milk the amount of tyrosine which may exist there either free or in peptide chain formation may be considerable.

SUMMARY.

1. Lactalbumin when fed as 12 and 18 per cent of a ration, carrying 2 per cent of the total protein in the form of nitrogen of unknown source in an alcoholic extract of wheat embryo to furnish the water-soluble vitamine, was found to be inadequate for growth.

2. When fortified with a weight of cystine equal to 1 per cent of that of the total protein, lactalbumin fed at an 18 and 12 per cent level was found to be of excellent nutritive value. Cystine, then, is the primary growth-limiting factor in that protein.

3. Lactalbumin, when fed at a 9 per cent plane of intake, even in the presence of 1 per cent of the total weight of the protein in the form of cystine, was found to be very deficient in nutritive value. However, when fortified in addition with tyrosine in amount equal to 5 per cent of the total protein, it was found to be of excellent nutritive value. Tyrosine, then, is the secondary growth-limiting factor in that protein.

4. Protein-free milk was found to have a total sulfur content of 0.2 per cent, the greater part of which is in organic form. The

results suggest that protein-free milk contains either cystine or organically bound sulfur which the animal organism can transform into cystine. Protein-free milk was found to give qualitative tests for tyrosine.

The author wishes to express his appreciation to Professor E. B. Hart and Professor H. Steenbock for many valuable suggestions and criticisms in connection with this work; also to Professor E. H. Farrington of the Dairy Department of the College of Agriculture for the splendid facilities offered him in the preparation of lactalbumin.

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OCCURRENCE OF INOSITE HEXAPHOSPHORIC ACID IN THE SEED OF THE SILVER MAPLE (ACER SACCHARINUM).

SIXTEENTH PAPER ON PHYTIN.

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WITH THE COLLABORATION OF W. L. KULP.

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(Received for publication, June 21, 1920.)

INTRODUCTION.

In an earlier paper from this laboratory[1] it was shown that the seeds of the silver maple are rich in organic phosphorus. The organic phosphorus compound was isolated as a barium salt but when the former paper was published this substance had not been identified. The completion of the investigation was delayed owing to the war and only recently were we able to resume this work.

In the first part of the investigation some maple seed was used which had been powdered and extracted with ether during the summer of 1917. The barium salt of the organic phosphorus compound isolated from this material was purified and crystallized. The product thus obtained appeared to be identical with tribarium inosite hexaphosphate or tribarium phytate preparations previously described.[2] On analysis results were obtained which agreed more closely with barium inosite pentaphosphate than with inosite hexaphosphate. It has been claimed by Rather[3] that the principal organic phosphorus compound existing in plant material is inosite pentaphosphoric acid and our results in this case apparently confirm this view. This was the first time, however, that we had isolated a substance having this composition.

[1] Anderson, R. J., *J. Biol. Chem.*, 1918, xxxiv, 509.
[2] Anderson, R. J., *J. Biol. Chem.*, 1914, xvii, 141, 151, 165.
[3] Rather, J. B., *J. Am. Chem. Soc.*, 1918, xl, 523.

A second preparation was therefore made of the barium salt from freshly powdered maple seed. This seed had been gathered at the same time as the former material. The carefully purified and crystallized barium salt was analyzed and its composition was found to agree closely with that of tribarium inosite hexaphosphate.

This difference in results could only be explained as due to partial hydrolysis of the inosite hexaphosphate in the powdered maple seed. The powdered material after being extracted with ether had been kept in a well stoppered bottle for about 2 years. Comparative analyses of the total and inorganic phosphorus in the old and the freshly powdered material fully verified the assumption that partial hydrolysis had occurred in the old powdered maple seed, although it had been stored under ideal conditions and it contained only a very small percentage of moisture.

Careful analyses were made to determine whether the enzyme, phytase, was present in maple seed. We employed the same method as was used in connection with phytase in wheat bran.[4] The small increase in inorganic phosphorus which was observed after digestion in water or dilute acids could hardly be ascribed to the influence of phytase. This enzyme must therefore be present in very minute amounts in maple seed, if it is not entirely absent.

EXPERIMENTAL.

First Preparation of Barium Salt.

The dry maple seed had been powdered and extracted with ether in 1917. Of this material 500 gm. were digested in 1,500 cc. of 1 per cent hydrochloric acid. The mixture was stirred occasionally and allowed to stand over night. It was then filtered through a layer of paper pulp on a Buchner funnel and washed with water until 2,000 cc. of extract were obtained. In order to bind the free hydrochloric acid 75 gm. of sodium acetate were added and the dissolved protein was precipitated by tannic acid. After filtering through paper pulp, 40 gm. of barium chloride in concentrated solution was added. The precipitate was filtered and washed in water.

[4] Anderson, R. J., *J. Biol. Chem.*, 1915, xx, 483.

After suspending this precipitate in water the barium was removed by adding a slight excess of sulfuric acid. The barium sulfate was filtered off and the filtrate precipitated by adding a concentrated solution of copper acetate. The copper precipitate was filtered, washed free of sulfates with water, suspended in water, and decomposed with hydrogen sulfide. The copper sulfide was removed and the filtrate was concentrated to about one-half its volume under reduced pressure at a temperature of from 40–45°C.

The resulting acid solution was precipitated with barium hydroxide, filtered, and washed in water. The precipitate was dissolved in very dilute hydrochloric acid and again precipitated with barium hydroxide. The substance was reprecipitated in this manner five times from dilute hydrochloric acid by barium hydroxide. The product was then precipitated three times from dilute hydrochloric acid by adding about one and one-half volumes of 95 per cent alcohol, washing free of chlorides with dilute alcohol after each precipitation. After the substance was precipitated with alcohol a fourth time, it was allowed to stand in contact with the mother liquor for several hours when it changed into the usual crystalline form of tribarium phytate. The crystalline precipitate was filtered and washed free of chlorides with dilute alcohol. It was then dissolved in dilute hydrochloric acid as before and the free acid nearly neutralized by adding cautiously a dilute solution of barium hydroxide until a faint precipitate remained which did not dissolve on shaking. The mixture was filtered and a concentrated solution of 20 gm. of barium chloride was added to the clear filtrate. A heavy crystalline deposit of the barium salt separated slowly on the bottom of the flask. After standing over night this was filtered off and washed with water until free from chlorides. The substance was then recrystallized four times from dilute hydrochloric acid by the addition of alcohol. It was filtered and washed free from chlorides in 30, 50, and 95 per cent alcohol. It was then washed in ether and dried in vacuum over sulfuric acid. The product was a snow-white, bulky, crystalline powder which weighed 3.4 gm. Its dilute nitric acid solution gave no reaction with silver nitrate or with ammonium molybdate showing the absence of chlorides and inorganic phosphate.

The substance was analyzed after drying at 110° in vacuum over phosphorus pentoxide.

0.3168 gm. substance gave 0.0383 gm. H_2O and 0.0864 gm. CO_2.
0.1915 " " " 0.1268 " $BaSO_4$ and 0.1156 gm. $Mg_2P_2O_7$.
Found: C, 7.43; H, 1.35; P, 16.82; Ba, 38.96 per cent.

The substance was again recrystallized three times from dilute hydrochloric acid by the addition of alcohol, filtered, washed, and dried as before.

After drying to constant weight as above it was analyzed.

0.2846 gm. substance gave 0.0335 gm. H_2O and 0.0735 gm. CO_2.
0.1838 " " " 0.1212 " $BaSO_4$ and 0.1103 gm. $Mg_2P_2O_7$.
Found: C, 7.04; H, 1.31; P, 16.73; Ba, 38.80 per cent.

The product was again recrystallized in the same manner, washed, and dried, and the following result obtained on analysis.

0.2527 gm. substance gave 0.0306 gm. H_2O and 0.0675 gm. CO_2.
0.1919 " " " 0.1265 " $BaSO_4$ and 0.1160 gm. $Mg_2P_2O_7$.
Found: C, 7.28; H, 1.35; P, 16.85; Ba, 38.79 per cent.

As recrystallization did not alter the composition of this substance it was undoubtedly pure. In crystal form and properties it appeared to be identical with the tribarium phytate isolated from other plant material but the analytical results do not agree with the theoretical composition of this salt. The carbon is too high and the phosphorus is too low. In fact, the analytical data agree more closely with the calculated composition of a mixed barium salt of inosite pentaphosphoric acid as is evident from the figures below.

For $(C_6H_{12}O_{21}P_5)_2\ Ba_5$ or $C_{12}H_{24}O_{42}P_{10}Ba_5 = 1,837$.
Calculated: C, 7.83; H, 1.30; P, 16.87; Ba, 37.40 per cent.
Found (1): C, 7.43; H, 1.35; P, 16.82; Ba, 38.96 " "
" (2): C, 7.04; H, 1.31; P, 16.73; Ba, 38.80 " "
" (3): C, 7.28; H, 1.35; P, 16.85; Ba, 38.79 " "

When it was found that this preparation differed in composition from tribarium phytate by containing a higher percentage of carbon and less phosphorus it was thought that the organic phosphorus compound might have undergone some spontaneous hydrolysis. The old powdered maple seed was therefore examined

for total and inorganic phosphorus in comparison with a sample of freshly powdered material. The result showed that 18 per cent of the total phosphorus was present as inorganic while in the freshly powdered material only 12 per cent of the total was inorganic phosphorus. It is evident therefore that some hydrolysis of the organic phosphorus had occurred in the old powdered maple seed.

Second Preparation of the Barium Salt From Freshly Powdered Maple Seed.

The freshly powdered maple seed, 800 gm., was digested for 5 hours in 3 liters of 1 per cent hydrochloric acid. The organic phosphoric acid was isolated as the barium salt and recrystallized in the manner described above. A snow-white, bulky, crystalline powder was finally obtained. The substance was analyzed after drying to constant weight at 105° in vacuum over phosphorus pentoxide.

0.3210 gm. substance gave 0.0352 gm. H_2O and 0.0779 gm. CO_2.
0.1705 " " " 0.1126 " $BaSO_4$ and 0.1044 gm. $Mg_2P_2O_7$.
Found: C, 6.61; H, 1.22; P, 17.06; Ba, 38.86 per cent.
For $C_6H_{12}O_{24}P_6Ba_3 = 1,066$.
Calculated: C, 6.75; H, 1.12; P, 17.44; Ba, 38.65 per cent.

The composition of this substance agrees closely with that of similar salts previously isolated from cotton-seed meal, oats, corn, commercial phytin, and wheat bran, which we have described.[5] The analyses of these preparations, which have been carefully purified and recrystallized many times, are in close agreement with the calculated composition of a tribarium salt of inosite hexaphosphoric acid.

Judging by the analytical results obtained with these crystalline barium salts, the composition of phytic acid is most accurately represented by the formula of inosite hexaphosphoric acid, $C_6H_{18}O_{24}P_6$; and not by the formula of inosite pentaphosphoric acid, $C_6H_{17}O_{21}P_5$ or by other formulas as stated by Rather[3] or by the formula $C_6H_{24}O_{27}P_6$ as stated by Posternak.[6]

[5] Anderson,[2] p. 190.
[6] Posternak, S., *Compt. rend. Acad.*, 1919, clxix, 37.

Hydrolysis of the Phytin from Maple Seed into Inosite and Phosphoric Acid.

The analyzed barium salt, 1.9 gm. was hydrolyzed with dilute sulfuric acid at 145° in an autoclave for about 2½ hours. The inosite was isolated in the usual way and after recrystallizing several times from water with the addition of alcohol 0.24 gm. of inosite was obtained which corresponds to 80 per cent of the theoretical yield. The inosite separated in the usual needle-shaped crystals; it gave the reaction of Scherer and it melted at 223° (uncorrected). Since the substance possessed the properties and gave the reactions of inosite the analysis was omitted.

Examination of Maple Seed for Phytase.

The freshly powdered maple seed was digested in the solvents mentioned in Table I as follows: Samples of 12.5 and 25 gm. were

TABLE I.

Inorganic Phosphorus in Maple Seed.

Solvent.	Inorganic phosphorus.
	per cent
Water	0.16
0.05 per cent HCl	0.14
0.10 " " "	0.12
0.20 " " "	0.11
0.20 " " acetic acid	0.11
0.30 " " HCl	0.10
1.00 " " "	0.09

macerated with frequent shaking for 24 hours in 242.5 cc. of the solvent; 7.5 cc. of concentrated nitric acid were then added and the mixture was well shaken and allowed to stand for ½ hour. It was then filtered and the inorganic phosphorus was determined in aliquots representing 5 and 10 gm. of the sample by precipitating with ammonium molybdate in the usual manner and then with magnesia mixture. After it was filtered and ignited, the residue was taken up in dilute hydrochloric acid and reprecipitated as magnesium ammonium phosphate, incinerated, and weighed as magnesium pyrophosphate.

The results shown in Table I were obtained.

The increase in inorganic phosphate obtained on digesting the material in water or in the very dilute acid was so slight as to preclude the presence of an active phytase in maple seed.

SUMMARY.

An investigation has been made of the organic phosphorus compound of maple seed.

From an old sample of powdered maple seed a crystalline barium salt was obtained which corresponded to a barium salt of inosite pentaphosphoric acid, but from freshly powdered maple seed a crystalline barium salt corresponding to tribarium inosite hexaphosphate was obtained.

Evidence is presented which shows that some spontaneous hydrolysis of the organic phosphorus compound in maple seed occurs when the powdered material has been stored for about 2 years.

Freshly powdered maple seed apparently does not contain any active phytase.

The principal organic phosphorus compound of maple seed is identical with the inosite hexaphosphoric acid which has been isolated from other plant material.

AMINO-ACIDS OF THE BLOOD AS THE PRECURSORS OF MILK PROTEINS.*

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(Received for publication, June 22, 1920.)

The formation of milk in the mammary glands of lactating cows is so rapid that a determinable reduction of the precursors of milk constituents in the blood which has passed through the glands may reasonably be expected. With this idea in mind, Kaufmann and Magne (1) have compared blood samples, obtained approximately simultaneously from the jugular and from the mammary (abdominal subcutaneous) veins of milking cows, and have found that the concentration of dextrose in the mammary blood is less than that in the jugular blood. They conclude that this difference is due to the utilization of dextrose by the mammary glands as the precursor of milk sugar.

In analogous experiments Meigs, Blatherwick, and Cary (2) have found that the phosphatide content of the mammary blood is lower than that of the jugular, and conclude that this material is the precursor of milk fat and milk phosphorus. Discussions of this method of determining the changes effected in blood as it passes through the mammary gland by comparing blood obtained from the mammary veins with that of other samples of venous blood (*e.g.* that from the jugular veins) and the reliability of results obtained in this way are to be found in the two articles to which reference has just been made.

It seems not unlikely *a priori* that the mammary gland may use the free amino-acids of the blood as the precursors of milk protein. Several fairly satisfactory methods of determining the amino-acid N of blood and blood plasma exist, and numerous determinations have been made with the blood of various species of animals.

* Published with the permission of the Secretary of Agriculture.

The amino-acid N of blood is uniformly very low compared with the protein content of milk. The conditions are, therefore, very favorable in this case for the success of experiments similar to those of Kaufmann and Magne. I have carried out such experiments with both dry and milking cows, comparing the concentration of amino-acid N in blood and plasma obtained approximately simultaneously from the jugular and mammary veins.

Analytical Method.

The amino-acid N in the amino-acid extract from the blood and plasma was determined by the HNO_2 method, using the smaller apparatus designed by Van Slyke (3). This amino-acid extract was prepared essentially according to the method recommended by Bock (4) with certain modifications. The procedure varied somewhat in the different experiments. That finally adopted was as follows:

Duplicate samples of 50 and 80 cc. of blood, and 80 and 100 cc. of plasma were measured out. 1 cc. of a freshly prepared 10 per cent aqueous extract of urease from jack bean meal[1] and 0.2 cc. of a 3 per cent solution of NaH_2PO_4 were added for each 10 cc. of sample. The mixture was allowed to stand about 2 to 3 hours at 30°C. to hydrolyze the urea.

The mixture was then run slowly into five volumes of boiling dilute acetic acid (0.0125 N for the blood and 0.015 N with the plasma samples), the original vessel was rinsed in, and the boiling with constant stirring continued for 5 minutes more. A volume of boiling water equal to about three times that of the sample was added, and the mixture boiled for about 5 minutes more. It was then transferred to a volumetric flask of about ten times the volume of the sample and brought to volume. (A drop or two of amyl or caprylic alcohol was used in the case of the blood samples to prevent a foam from interfering in bringing them to volume.) The mixture was then shaken vigorously for some time, the temperature taken, and the contents were filtered through a folded filter paper into a flask containing 2 gm. of finely powdered kaolin per 100 cc. of mixture to be filtered. The filtrate was pressed out of the residue on the paper as completely as convenient. It was then shaken up several times with the kaolin and, after standing for some time, was filtered through a No. 42 Whatman filter paper and then refiltered through a No. 44 Whatman paper. The filtrate thus obtained was perfectly clear and colorless. The temperature was taken and as large an aliquot as possible taken out.

[1] One part of finely powdered jack bean meal and ten parts of water were shaken together several times during an hour, centrifuged, and the supernatant liquid was pipetted off and used.

This aliquot was concentrated rapidly to about 10 cc. by boiling in a Kjeldahl flask through which a stream of air was passing. Only a slight amount of whitish sediment appeared. The concentrated solution with the sediment was transferred to a 50 cc. volumetric flask, 5 cc. of an aqueous solution of trichloroacetic acid[2] were added, and the whole was brought to volume. It was then poured upon 2 gm. of kaolin, shaken several times during an hour, centrifuged, and filtered through a No. 44 Whatman paper. The filtrate was perfectly clear. A 45 cc. aliquot was taken out.

This second aliquot was heated in a flask in a pan of boiling water for 3 hours. Either a long necked flask or a rubber stopper with a piece of glass tubing as a reflux was used to prevent concentration of the solution. After thus decomposing the trichloroacetic acid, the solution was transferred to an aerating cylinder. 4 cc. of a 10 per cent suspension of calcium hydroxide, a drop or two of a 0.5 per cent aqueous suspension of phenolphthalein, and about four drops of caprylic alcohol were added. The aeration was continued for about 3 hours. The mixture was then filtered into a small beaker, the cylinders and paper were washed with ammonia-free water, the filtrate and washings together neutralized with 50 per cent acetic acid, and an excess of two or three drops of this acid added.

The solution was then concentrated on an electric hot plate at just below the boiling point, and finally brought to 10 or 15 cc. in a volumetric flask. This was generally refiltered before running on the amino-acid apparatus.

In determining the amino-acid N on the Van Slyke apparatus with the extracts prepared as above, the evolution of N was never complete in the time allowed for the deamination of the α-amino N, which was 5 minutes at 25°C. In several different analyses of the same amino-acid extract, in which deamination times of 5, 10, and 15 minutes were allowed, it was found that the evolution of N (corrected for reagents) during the third 5 minutes was practically the same as that during the second 5 minutes. Hence it was assumed that this slowly evolved N came off at practically the same rate during the first 5 minutes, and a correction for it was accordingly made in all the analyses of extracts prepared as above described. Several determinations were made on each extract, allowing the shorter deamination time (sufficient for the reaction of the α-amino N to go to completion, and for about 95

[2] 100 cc. of solution contained 50 gm. of trichloroacetic acid. The protein would thus be precipitated in a 5 per cent concentration of trichloroacetic acid. In a private communication Dr. Bock recommends this concentration instead of that in his article (4).

per cent of the ϵ-amino group of lysine to come off), and several allowing a deamination time twice as long. For the slowly evolved N correction was made essentially as suggested by Van Slyke and Meyer (5), by subtracting the average (corrected for reagents) of the shorter runs from that of the longer and subtracting this difference from the former.

During the determination on the amino-acid apparatus with all the samples to be compared the temperature was kept constant. The samples for analysis were measured into the apparatus with a 2 cc. Ostwald pipette; and the various determinations of the jugular and mammary samples to be compared were run alternately on the machine, so as to keep the conditions as nearly comparable as possible.

Throughout the whole procedure a jugular sample and a corresponding mammary sample of the same size were run as a pair under as nearly the same conditions as possible.

The amino N was determined in the urease extract used in each experiment by the same method as above, and correction was made for it accordingly.

Each datum, therefore, with each of the duplicate samples of blood or plasma given in Table II of this article, is the average of several determinations on the amino-acid apparatus corrected for (1) the reagents used, (2) any slowly evolved N, and (3) the amino N of the urease used.[3]

EXPERIMENTAL.

My results are summarized in Table I. More detailed data are given in Table II and its accompanying note.

[3] The figures for amino-acid N that I have obtained from the blood and plasma of cows are considerably lower than those of Bock (6) and Okada (7) from beef animals, although they used an analytical procedure essentially the same as that which I have followed, but they do not state what corrections enter into the calculation of their results or whether they corrected for the amino-acid N of the soy bean meal that they used.

TABLE I.

Average Amino-Acid N in Blood and Plasma, and Difference in Plasma Samples.

Experiment No. Cow No. Date.	Average amino-acid N per 100 cc.				Difference in plasma samples.		Milk yield daily.
	Blood.		Plasma.		Amount.	Per cent of jugular.	
	Jugular.	Mammary.	Jugular.	Mammary.			
1919	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*liters*
I 51 Sept. 19			2.69	1.78	0.91	33.8	12.77
II 51 Sept. 26			3.31	2.34	0.97	29.3	12.55
III 64 Oct. 15	4.49	4.32	2.49	1.92	0.57	22.9	8.70
IV 51 Nov. 28	4.01	3.83	2.38	1.99	0.39	16.4	9.88
V 51 Dec. 8	4.19	3.91					9.69
VI 51 Dec. 15			2.47	1.87	0.60	24.3	9.65
1920							
VII 56 Feb. 2	3.99	3.92	2.14	2.20			Dry.
VIII 63 Feb. 17			2.40	2.51			"
IX 227 Mar. 2	5.34	.58	3.02	2.35	0.67	22.2	25.14

TABLE II.

Data from Experiments on Both Dry and Milking Cows.

Experiment No. Cow No. Date.	Corpuscle volume.		Amino N per 100 cc.				Milk yield daily.	Crude protein N of daily diet.
			Blood.		Plasma.			
	Jugular.	Mammary.	a. Jugular 1.* b. Jugular 2.* c. Average.	a. Mammary 1. b. Mammary 2. c. Average.	a. Jugular 1. b. Jugular 2. c. Average.	a. Mammary 1. b. Mammary 2. c. Average.		
1919	*per cent*	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*	*liters*	*gm.*
I					a. 2.66	a. 1.67		
51					b. 2.72	b. 1.89	12.77	188.1
Sept. 19					c. 2.69	c. 1.78		
II					a. 3.47	a. 2.38		
51	32.1	30.3			b. 3.15	b. 2.31	12.55	188.1
Sept. 26					c. 3.31	c. 2.34		
III					a. 2.30	a. 1.78		
64	36.75	36.0	a. 4.49	a. 4.32	b. 2.68	b. 2.07	8.70	162.8
Oct. 15					c. 2.49	c. 1.92		
IV			a. 3.99	a. 3.85	a. 2.42	a. 2.10		
51	31.6	31.6	b. 4.03	b. 3.81	b. 2.34	b. 1.89	9.88	164.7
Nov. 28			c. 4.01	c. 3.83	c. 2.38	c. 1.99		

* In Experiments I to VI inclusive, the urea and ammonia were not removed and the samples for duplicate analyses on the amino-acid apparatus were measured in from the burette on the side of the apparatus. In Experiments I to IV inclusive, with the exception of the plasma samples of Experiment IV, the filtrate from the heat-acetic acid coagulation was not shaken with kaolin.

In Experiments I to III inclusive, the deamination times were 4 and 8 minutes at temperatures of 21–25°C. In Experiments IV and V the deamination times were $5\frac{1}{2}$ and $8\frac{1}{4}$ minutes with temperatures from 18.5–24°C. In Experiment VI, the deamination times were 4 and 8 minutes at 27–28°C., and in Experiments VII to IX, inclusive, the times were 5 and 10 minutes at temperatures from 25–27°C. In Experiments I to VI inclusive the several analyses on the amino-acid apparatus of each blood or plasma duplicate were run off consecutively instead of alternating with the sample to be compared with it, as described above.

TABLE II.—*Continued.*

Experiment No. Cow No. Date.	Corpuscle volume.		Amino N per 100 cc.				Milk yield daily.	Crude protein N of daily diet.
			Blood.		Plasma.			
	Jugular.	Mammary.	a. Jugular 1.* b. Jugular 2.* c. Average.	a. Mammary 1 b. Mammary 2 c. Average.	a. Jugular 1. b. Jugular 2. c. Average.	a. Mammary 1 b. Mammary 2 c. Average.		
1919	*per cent*	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*	*liters*	*gm.*
V								
51			a. 4.19	a. 3.91			9.69	164.7
Dec. 8								
VI					a. 2.47	a. 1.85		
51	32.3	31.4			b. 2.48	b. 1.89	9.65	164.7
Dec. 15					c. 2.47	c. 1.87		
1920								
VII					a. 2.18	a. 2.16	Dry since	
56	30.8	30.4	a. 3.99	a. 3.92	b. 2.10	b. 2.25	Oct.,	127.0
Feb. 2					c. 2.14	c. 2.20	1918.	
VIII					a. 2.44	a. 2.52	Dry since	
63	35.2	33.9			b. 2.37	b. 2.51	July 12,	82.6
Feb. 17					c. 2.40	c. 2.51	1919.	
IX			a. 5.39	a. 4.65	a. 3.04	a. 2.35		
227	33.0	30.7	b. 5.30	b. 4.51	b. 3.00	b. 2.35	25.14	388.0
Mar. 2			c. 5.34	c. 4.58	c. 3.02	c. 2.35		

Details of Experiments.

Table II gives the details and data obtained in the experiments that I have tried with both dry and milking cows. The milk yield is the average of 3 days, including one before and one after the day on which the samples of blood were taken. All the cows were given their morning feed at the customary time, between 4 and 5 a.m. The samples of blood were generally taken between 8.30 and 9.15 a.m. The sample from the mammary vein was always taken first.

The area over the vein was bathed with a 5 per cent solution of phenol, sometimes some of the hair was clipped away, and then a trocar and cannula were inserted into the vein. Generally the cow

was not disturbed more than about 2 minutes before the flow of blood was secured. About 1 to 3 minutes were required to collect the mammary sample. About 2 minutes intervened before the flow of jugular blood was obtained and 1 to $2\frac{1}{2}$ minutes were required to collect this sample. The samples varied from 200 to 650 cc. There was no relation between the size of sample and the data obtained. The samples were collected in bottles containing 0.1 gm. of dry sodium oxalate per 100 cc. of blood to be taken.

The plasma was obtained immediately by centrifuging the blood for 20 minutes at 3,600 revolutions per minute. The corpuscle volume was noted on a graduated centrifuge tube.

The analytical procedure was that described above except as indicated in the footnote to Table II.

Table II includes all the data that I have obtained except: (1) the first two experiments that I made, in which the analytical methods subsequently used were not fully worked out; (2) one subsequent experiment in which my procedure was varied and in which the duplicates did not agree at all; and (3) the blood determinations of Experiment VI, which were made by several different methods that did not give concordant results. The duplicate blood determinations in Experiments III, V, and VII were lost.

I was unable to note any relation between the apparent disturbance of the cows and the variations in the amino-acid N of the blood and hence have omitted the details bearing upon this point.

DISCUSSION.

In the six experiments with milking cows, the amino-acid N of the mammary plasma is decidedly lower than that of the jugular, the differences varying from 0.39 to 0.97 mg. per 100 cc. of plasma in the different experiments. In the four experiments with milking cows in which the amino-acid N of the whole blood was determined it is lower in the mammary than in the jugular blood, although the differences are sometimes small. In the experiments with dry cows, the jugular and mammary samples of both blood and plasma are practically identical.

In Table III the maximum absolute difference between the duplicates is compared with the difference between the jugular and mammary averages in the case of the plasma determinations in each experiment.

In all the experiments on milking cows the differences in the amino-acid content of the jugular and mammary samples of plasma are greater than the maximum differences between the duplicates. The results, as a whole, furnish conclusive evidence for the view that the mammary plasma of milking cows contains decidedly less amino-acid N than the jugular plasma.

The differences between the amino-acid N contents of the jugular and mammary samples of blood in my experiments were generally less, not only relatively but absolutely, than the corresponding differences in the samples of plasma. The greater plasma

TABLE III.

Maximum Absolute Difference between Duplicates Compared with Difference between Jugular and Mammary Plasma Averages.

Experiment No.	Maximum difference in plasma duplicates.	Difference between jugular and mammary plasma averages.		Milk yield daily.
		Mammary less.	Mammary greater.	
	mg.	*mg.*	*mg.*	*liters*
I	0.22	0.91		12.77
II	0.32	0.97		12.55
III	0.38	0.57		8.70
IV	0.21	0.39		9.88
V	No plasma determinations.			
VI	0.04	0.60		9.65
VII	0.09		0.06	0.00
VIII	0.07		0.11	0.00
IX	0.04	0.67		25.14

differences probably mean that the amino-acids of the blood corpuscles take no part in the interchange which goes on between the blood and the mammary gland and that there has been no migration of amino-acids from the corpuscles to the plasma of the samples of blood between the time that they were collected and the time that they were centrifuged. In view of these circumstances, I shall consider only the amino-acids of the plasma in attempting to compare the reduction of the concentration of these compounds in the mammary samples with the amount of protein secreted by the mammary gland in a given time.

There have been no definite determinations of the flow of blood through the mammary gland. It has been roughly estimated by

Meigs, Blatherwick, and Cary (2) from the data of Kaufmann and Magne (1) that with a cow giving 10 liters of milk daily about 3.5 liters of blood pass through the mammary glands per minute. The largest difference in amino-acid N that I have determined between the jugular and mammary plasma was nearly 1 mg. per 100 cc. in Experiment II with No. 51. She was giving 12.55 liters of milk daily. The corpuscle volume of the jugular blood was about 32 per cent. If it is assumed that the blood flow through her gland was at the rate of 3.5 liters per minute, this would mean that the flow of plasma amounted to about two-thirds of this figure, or 2.3 liters per minute.

If milk secretion goes on continuously throughout the 24 hours, as suggested by Meigs, Blatherwick, and Cary (2), about 33 gm. of α-amino-acid N, according to the result in this experiment, would be abstracted daily by the mammary gland from the plasma of the blood perfusing it. The α-amino N of the milk proteins is about 70 per cent of their total N. Hence the amino N thus taken up from the plasma of the blood daily would be equivalent, in this experiment, to about 300 gm. of milk protein or a protein content of the milk of 2.4 per cent. This estimate is very rough. It indicates, however, that the differences are roughly of approximately sufficient magnitude to account for the secretion of milk protein.

An interesting feature of the work which remains to be discussed is the result obtained in Experiment IX. The subject of this experiment was giving about 25 liters of milk daily—from two to two and a half times that given by the other milking cows. But in spite of this, the absolute decrease in the amino-acid N of the mammary plasma was practically the same as the average absolute decrease for the other milking cows. This result may be interpreted to mean that as a general rule the flow of blood through the mammary gland is proportional to the milk yield. There is a good deal of evidence to support this view (2).

The CO_2 Capacity of Jugular and Mammary Plasma.

Dr. N. R. Blatherwick has determined the CO_2 capacity of the jugular and mammary plasma in some of the experiments reported above. With his permission, I am including these data in Table IV.

As far as the above data go, it would seem that the following observations are justified.

1. The CO_2 capacity of the plasma of the blood from the inactive mammary gland is somewhat greater than that of the plasma from the jugular blood.

2. The difference in CO_2 capacity as between the jugular and mammary plasma of milking cows is relatively greater than that in the case of the non-lactating cow.

TABLE IV.

CO_2 Capacity of Jugular and Mammary Plasma.

Experiment No. Cow No. Date.	CO_2 capacity in volumes.		Milk yield daily.
	Jugular.	Mammary.	
1919	*per cent*	*per cent*	*liters*
III 64 Oct. 15	64.5	69.2	8.70
IV 51 Nov. 28	65.5	70.2	9.88
1920			
VIII 63 Feb. 17	59.5	61.4	0.00
IX 227 Mar. 2	69.1	72.0	25.14

3. If we compare the differences in CO_2 capacity obtained from Nos. 51 and 64 with that from No. 227, there are two possibilities: The CO_2 capacity of the arterial blood was already high in the case of No. 227 as indicated by the CO_2 capacity of the jugular plasma, and it may be that the increase in CO_2 content of the blood passing through the mammary gland did not therefore effect relatively so great a change in CO_2 capacity; or, second, it may be that the metabolism indicated by this increased CO_2 capacity in the mammary plasma is not proportional to the milk yield and

therefore in the high producing cow is less relative to the flow of blood through the gland.

A calculation of the CO_2 output of the mammary gland from the data at hand would involve several more or less unsupported assumptions and hence would not be very reliable.

SUMMARY.

1. The amino-acid N of the blood and blood plasma obtained from the mammary vein in non-lactating cows is practically the same as that obtained from the jugular vein.

2. The amino-acid N of blood and blood plasma obtained from the mammary vein in milking cows is decidedly lower than that obtained from the jugular. The mammary plasma has been found to contain from 16 to 34 per cent less amino-acid N than the jugular plasma.

3. The amino-acids thus picked up by the mammary gland from the plasma of the blood perfusing it are sufficient to account for the proteins of the milk and are undoubtedly the precursors of these milk constituents.

4. Data are presented on the CO_2 capacity of the plasma of blood from the jugular and mammary veins of milking and dry cows.

In conclusion, I desire to express my sincere appreciation of the suggestions and kindly interest in the work of Dr. E. B. Meigs and Dr. N. R. Blatherwick, of this laboratory, and Mr. T. E. Woodward, Superintendent of the Dairy Division Farm at Beltsville, Md., where this work was conducted.

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STUDIES ON CARBOHYDRATE METABOLISM IN RABBITS.

I. OBSERVATIONS ON THE LIMITS OF ASSIMILABILITY OF VARIOUS CARBOHYDRATES.*

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In the advertising literature of a well known proprietary food for infants and invalids, the statement is made that this product, which is said to be a mixture of dextrin and maltose prepared by the action of malt diastase on the starch of cereals, is "100 per cent more assimilable than either milk or cane sugar." Upon investigation it was found that this statement was based on a paper by Lovett, Morse, and Talbot,[1] who quoted the limits of assimilation of the different sugars recorded by various investigators as follows:

"Grape Sugar: In babies, about 5 grams per kilo (Langstein and Meyer).
Grape Sugar: In one-month baby, 8.6 grams per kilo (Greenfield).
Galactose: No accurate data.
Levulose: Lower for babies than adults. One gram per kilo (Keller).
Maltose: Over 7.7 grams per kilo (Reuss).
Lactose: 3.1–3.6 grams per kilo (Grosz).
Cane Sugar: About the same as lactose (Reuss)."

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* The data in this paper were taken from the dissertation presented by Martha R. Jones for the degree of Doctor of Philosophy, Yale University, 1920. Part of the expense of the investigation was defrayed by a grant from the Russell H. Chittenden Research Fund for Physiological Chemistry.

† Research Fellow (1919–20) of the Boston Branch of the Association of Collegiate Alumnæ.

[1] Lovett, R. W., Morse, J. L., and Talbot, F. B., *Boston Med. and Surg. J.*, 1911, clxiv, 852.

A perusal of the scanty literature on the subject of alimentary sugar tolerance showed that the figures quoted had been obtained from a comparatively few isolated experiments which had been performed by various investigators using different methods and under totally different conditions. Since data of this sort are in no way comparable, more evidence seemed necessary before definite conclusions could be drawn in regard to the relative assimilability of sugars. In view of the very extensive use of carbohydrates of various kinds in dietaries of infants, and the uncertainty of the medical profession in regard to their relative values, the present investigation was planned to study the question under conditions involving as few variables as possible.

In clinical practice sugar tolerance is considered to be the largest number of grams of glucose which can be ingested at one time without causing glycosuria. The alimentary method of determining tolerance obviously has its limitations, since the entry of the sugar into the blood depends not only upon the motor power of the stomach and intestines, but also on the rate of absorption which varies with a wide range of conditions. Real tolerance, as Woodyatt, Sansum, and Wilder[2] remind us, depends upon the rate at which the tissues are able to abstract glucose from the blood and utilize it. Sugar introduced directly into the circulation does not have to undergo absorption, hence the rate of injection can be so regulated that it is just equal to the rate at which the blood and tissues will bind and hold the sugar without an overflow through the kidneys. From this standpoint, glucose tolerance signifies the largest number of grams per kilo of body weight per hour which can be injected into a vein without causing glycosuria. Since the sugars in which we are primarily interested are constituents of foods, it seemed that the oral method of studying tolerance was preferable for our purposes, and hence this method was selected.

Feeding and Analytical Technique.

Rabbits were used: seven females and ninety-six males. They were divided into groups and kept in large pens. Free access to corn, oats, and water was had at all times, and fresh cab-

[2] Woodyatt, R. T., Sansum, W. D., and Wilder, R. M., *J. Am. Med. Assn.*, 1915, lxv, 2067.

bage, in limited quantities, was fed daily. This diet was always relished and was sufficiently well balanced to keep the animals in good nutritive condition over a long period of time. One rabbit was kept during the entire experimental period of 9 months and within that time received forty doses of sugar without showing ill effects.

The rabbits were usually taken from the pens on the afternoon before they were to be used, and placed in metabolism cages in which there was an abundance of oats and water. The next morning each animal was weighed and the normal urine collected by compressing the abdominal wall over the bladder. The urine was tested qualitatively for reducing substances with Benedict's reagent, and if the reaction was negative the rabbit was fastened on the animal board and the sugar solution administered by means of a stomach sound. Great care was exercised to excite the animals as little as possible, and with very few exceptions they neither offered resistance nor exhibited fear.

Sugar solutions were made up to a volume of 50 cc. except in a few cases where the concentration was so great that the liquid could not run through the tube. In such cases the volume was increased to as much as 65 cc. After the administration of the sugar the rabbits were returned to their cages where they had free access to oats, cabbage, and water. Since cabbage contains some sugar, this probably introduced a source of error, but the amount eaten after the ingestion of the carbohydrate solution was usually quite small, and hence the error was negligible. After an interval of 3½ hours the urine was again collected and tested qualitatively with Benedict's reagent. Since we were not concerned with mere traces of reducing substances, all tests which were not definitely positive were recorded as negative. As used in these experiments, Benedict's reagent showed a positive reduction when glucose was present in a concentration of 0.08 per cent. Early in the investigation the animals were always kept in their metabolism cages for 24 hours after the ingestion of the carbohydrate solution and the total urine voided during this time was tested, but it was invariably found that if sugar appeared at all the greater portion was excreted during the first 3½ hours; hence, this procedure was discontinued. Frequent confirmatory osazone and fermentation tests were made. If the

test was negative, the dosage of sugar was increased 1 to 3 gm. per kilo until sugar appeared in the urine. *The smallest amount of carbohydrate which resulted in glycosuria was recorded as the assimilation limit.* In sucrose experiments and frequently after the ingestion of maltose and dextrin the urine was made acid (1 per cent) with HCl, hydrolyzed for 30 minutes in a boiling water bath, cooled, neutralized, and tested again for sugar. Doses were repeated at intervals of 48 hours until each animal had received three. During this time the rabbit was kept in its metabolism cage and at the end of the period was returned to the large pen where it remained for a week or more. In order to determine whether repeated doses of sugar had any effect upon tolerance, some of the animals were divided into groups and the order of administration varied.

Early in the investigation sucrose solutions were given and much difficulty was experienced in obtaining consistent results. It was noticed that unfiltered normal urine would often reduce Benedict's solution while the filtered urine would not. All urine was then filtered through ordinary filter paper. Frequently the first few drops of the filtrate were tested and these sometimes would reduce Benedict's solution while the mixed filtrate before and after inversion would not. Upon investigation it was found that after repeated doses of sucrose, an enzyme was excreted in the urine which acted upon the starch in the filter paper and produced sufficient sugar to cause a reduction of Benedict's solution. Following this discovery the urines were heated on a water bath for 2 or 3 minutes before filtering and the procedure continued as before. No discrepancies were observed after this precaution. In making so large a number of reduction tests with Benedict's solution, more uniform results were obtained by heating the tubes in boiling water for 6 minutes than by boiling each separately over a free flame; hence, this method was adopted.

Feeding Experiments with Carbohydrates.

Sucrose.—The limit of assimilability of sucrose was determined in twenty-two animals, the doses which just produced glycosuria ranging from 4 to 9 gm. per kilo. Thirteen of these animals had a marked increase in reducing sugar in the urine after inversion, indicating the presence of sucrose. Two rabbits which excreted reducing sugar after the ingestion of 6 and 7 gm. per kilo of sucrose, respectively, excreted sucrose, as such, when the dosage was increased 1 gm. per kilo each. To another rabbit in which

the assimilation limit was 9 gm. per kilo, sucrose appearing in the urine, a dose of 10.5 gm. per kilo was administered, the apparatus being so adjusted that the animal received 1 cc. of solution or 0.23 gm. per kilo of sugar per minute. Now the urine test was positive, but only reducing sugar was present. Eight of the fifty-eight animals receiving sucrose developed diarrhea, but in seven of these cases the dose exceeded the assimilation limit. Six of the eight cases of diarrhea were accompanied by sucrosuria. One animal had diarrhea after the ingestion of a dose smaller than the assimilation limit, but at a later time a much larger dose did not result in intestinal disturbances. No effort was made in any of these cases to determine the presence of sugar in the feces.

TABLE I.

Animal No.	Limits of assimilability per kilo.	
	Sucrose.	Inverted sucrose.
	gm.	gm.
4	5	5
19	8	8
21	5	7 (Diarrhea.)
22	8 (Diarrhea.)	8 −
24	8	8 +
51	8 −	8 (Diarrhea.)
52	6	8 +
65	7	9
66	5	8
68	9	9
71	6	7
72	6	7
75	6	7
77	6	8

Plus and minus signs, wherever used, indicate that the limit of assimilability was not determined but was greater or less, respectively, than the figure given.

Inverted Sucrose.—Sucrose solutions were made up to a volume of 50 cc., made acid (1 per cent) with HCl, and heated on a boiling water bath for 30 minutes. They were then cooled, neutralized with NaOH, made up to a volume of 50 cc. with distilled water, and administered as previously described. The limits of assimi-

lability of the invert sugar ranged from 5 to 9 gm. per kilo in eleven animals.

Table I shows the limits of assimilability of sucrose and inverted sucrose in the same animals.

In only one case, Rabbit 22, was the assimilability of the inverted sucrose less than that of sucrose. In fact, with the exception of Rabbit 22 in which the limit of assimilability of inverted sucrose was not determined, the average tolerance for inverted sucrose in the animals given above was more than 7.6 gm. per kilo, while the same animals had a tolerance of less than 6.5 gm. for sucrose. Three rabbits (Nos. 4, 19, 68) showed the same tolerance for inverted sucrose as for sucrose, while the remaining ten exhibited a greater tolerance for the inverted sugar. Two of the animals which had the same tolerance for sucrose as for inverted sucrose, had only reducing sugar in the urine after the ingestion of sucrose. Evidently, in these cases, the sugar was inverted in the intestines before absorption. Three rabbits which had a greater tolerance for inverted sucrose than for sucrose had sucrosuria. It would seem from these experiments that sucrose may be quite as rapidly absorbed as the monosaccharides, and since the rabbit has no sucrase in its normal serum the tissues are unable to utilize it and it is excreted by the kidneys.

Levulose.—The limit of assimilability of levulose was determined in nine animals, the doses ranging from 3 to 10 gm. per kilo. Rabbit 24 received 14 gm. per kilo without sugar appearing in the urine. Diarrhea occurred only once after levulose ingestion, the animal receiving 9 gm. per kilo, but no sugar appeared in the urine.

Table II shows the relative assimilability of sucrose and levulose in the same animals.

Seven of the nine rabbits showed a tolerance for levulose equal to or greater than that for sucrose, while two had a slightly greater tolerance for sucrose.

Glucose.—In fourteen rabbits the limits of assimilability of glucose ranged from 10 to 16 gm. per kilo. Six of the eighteen animals which received glucose developed diarrhea, but in three of these cases the assimilation limit was exceeded. In one case a dose of 10 gm. per kilo resulted in diarrhea without sugar

appearing in the urine. A few days later this animal received a dose of 12 gm. per kilo. Sugar appeared in the urine but the feces were normal. Three animals had glucosuria without diarrhea after the ingestion of 16 gm. per kilo.

Table III gives the comparison of glucose tolerance with that of sucrose and levulose in the same animals.

TABLE II.

Animal No.	Limits of assimilability per kilo.	
	Sucrose.	Levulose.
	gm.	gm.
4	5	3
19	8	8
21	5	9
22	8	10
23	4 −	7 +
24	8	14 +
40	5	5 −
49	8 −	9 +
52	6	10

TABLE III.

Animal No.	Limits of assimilability per kilo.		
	Sucrose.	Glucose.	Levulose.
	gm.	gm.	gm.
4	5	14	3
19	8	12	8
21	5	12 (Diarrhea.)	9
23	4 −	12	7 +
24	8	14 (Diarrhea.)	14 +
40	5	12 +	5 −
70	7	12	(?)

An examination of Table III shows that glucose tolerance was invariably higher than that of sucrose, and with one exception (Rabbit 24) was higher than that of levulose.

Lactose.—Four rabbits received doses of lactose ranging from 6.4 to 9 gm. per kilo without the appearance of sugar in the urine or diarrhea. Since it was found impossible to keep this quantity of sugar in solution in a volume of 50 cc., we decided to discon-

tinue the test rather than introduce another variable into our experiments.

Maltose.—The limit of assimilability of maltose was determined in six animals. Two showed glycosuria after the ingestion of 10 and 14 gm. per kilo respectively, although the average tolerance of the remaining four was 19.5 gm. Doses ranging from 16 to 20 gm. per kilo were fed to seven rabbits without sugar in the urine or diarrhea resulting.

Dextrin.—Nineteen rabbits received doses of dextrin ranging from 15 to 20 gm. per kilo without exhibiting glycosuria, while the limit of assimilability was determined in only four. Rabbit 15 had a dextrin tolerance of 5.5 gm. per kilo. This animal exhibited relatively low tolerance for all carbohydrates and was the only one in which the limit of assimilability of dextrin was as low as that of sucrose. The average tolerance of the remaining three rabbits was 16.1 gm. per kilo. Occasionally the urine collected after the ingestion of dextrin was hydrolyzed and tested for sugar, but at no time was there an appreciable increase in reduction.

Dextri-Maltose.—The limits of assimilability of the carbohydrates ordinarily used in infant feeding having been determined, it seemed desirable to study the effect of various mixtures upon tolerance. Two commercial products, dextri-maltose (Mead's) and malt sugar (Borcherdt's), were selected. Dextri-maltose is reported[3] to contain 52.0 per cent maltose, 41.7 per cent dextrin, 2.0 per cent sodium chloride, and 4.3 per cent moisture, and is used as a food for infants and invalids. This preparation was fed to thirty-nine rabbits, twenty-one of which had an average tolerance of 14.5 gm. per kilo, while in eighteen the limit was not determined. Two animals received doses of 21 gm. per kilo without the appearance of sugar in the urine. Several tolerated as much as 18 gm. per kilo without glycosuria, while the limit of assimilability in one was only 6 gm. per kilo and in two others, less than 9. Rabbit 2 received a dose of 13 gm. per kilo, and 45 minutes later a second dose, 14 gm. per kilo, was administered. Neither the urine collected immediately before nor 3½ hours after the ingestion of the second dose con-

[3] New and Non-official Remedies, 1919.

tained sugar. Several weeks later, this animal tolerated 22 gm. per kilo of this preparation without exhibiting glycosuria.

Malt Sugar.—Borcherdt's malt sugar which is also a food for invalids and infants is reported[3] to contain 87.0 per cent maltose, 4.35 per cent dextrin, 1.9 per cent inorganic salts, and 4.4 per cent protein. This product was used with the view of ascertaining whether different proportions of dextrin and maltose would have any effect upon the assimilability of the mixture. Twenty-seven animals received doses of this preparation. In eleven rabbits the

TABLE IV.

Animal No.	Limits of assimilability per kilo.	
	Dextri-maltose.	Malt sugar.
	gm.	*gm.*
4	13.0	16.0
7	9.5	12.5
9	11.5	16.0
10	19.0	20.0 +
12	11.0	15.5 +
14	9.0	13.5
15	6.0	8.0
16	9.5	12.0
18	11.0	11.0 +
19	12.0	18.0
21	9.0 −	16.0
23	13.0	15.0
34	18.0 −	18.0 +
38	18.0	18.0 +
39	18.0	19.0

tolerance ranged from 8 to 19 gm. per kilo, although in twelve other animals doses ranging from 15 to 20 gm. per kilo did not result in glycosuria.

Table IV shows the relative assimilability of the two preparations in the same animals.

An examination of Table IV shows that all fifteen of the animals which received doses of the two commercial preparations had a greater tolerance for malt sugar than for dextri-maltose. Rabbit 19 assimilated 6 gm. per kilo more of malt sugar than of dextri-maltose, no diarrhea resulting in either case, while Rabbit 21 tolerated at least twice as much of the former as of the latter.

Numerous investigators have shown that the addition of meat to sugar increases the tolerance for the latter, and the presence of protein in malt sugar might account for its greater assimilability. Five cases of diarrhea without glycosuria occurred after the ingestion of malt sugar, the doses ranging from 18 to 19 gm. per kilo. Six cases of diarrhea resulted after the ingestion of dextri-maltose, the doses ranging from 11 to 20 gm. per kilo. With one exception, no sugar appeared in the urine. Rabbit 35 had three doses, 15, 18, and 20 gm. per kilo, respectively, of dextri-maltose, all of which resulted in diarrhea, but sugar appeared in the urine only after the ingestion of 20 gm. per kilo. From these experiments, it would seem that the rate of absorption of the two preparations is relatively slow.

Maltose + Dextrin.—The next step in the investigation was the determination of the tolerance for known mixtures of carbohydrates. The limits of assimilability of dextrin and maltose mixed in the proportion of 1:1 ranged from 13 to 16 gm. per kilo in four animals, the average tolerance being approximately the same as that for dextri-maltose.

TABLE V.

Animal No.	Limits of assimilability per kilo.			
	Maltose.	Dextrin.	Dextri-maltose.	Maltose + dextrin.
	gm.	gm.	gm.	gm.
4	17.5 +	16.0 +	13.0	14.0
9	20.0 −	11.5 +	11.5	
10	19.0		19.0	
12	21.0	8.0 +	11.0	
15	10.0	5.5	6.0	
19		18.0 +	12.0	
21		18.0	9.0 −	
23	18.0		13.0	13.0
34	20.0 +	17.5 +	18.0 −	
35	20.0 +	18.0 +	20.0	
65	16.0 +			16.0
66	16.0 +			16.0

Table V shows the relative assimilability of maltose, dextrin, dextri-maltose, and a mixture of equal parts of maltose and dextrin in the same animals.

It appears that the tolerance for dextri-maltose and the dextrin-maltose mixture was less than that for either pure maltose or pure dextrin.

Sucrose + Dextrin.—Since rabbits have a very high tolerance for dextrin and a relatively low tolerance for sucrose, it was expected that the assimilation limit of a mixture of the two carbohydrates would lie somewhere between the limits of dextrin and sucrose; hence, a series of experiments was planned to study the behavior of sucrose-dextrin mixtures.

TABLE VI.

Animal No.	Limits of assimilability per kilo.		
	Sucrose.	Sucrose + dextrin.	Dextrin.
	gm.	gm.	gm.
4	5.0	6.0	16.0 +
19	8.0	7.0 −	18.0 +
21	5.0	8.0	
23	4.0 −	8.0	
24	8.0	8.0	
35	8.0 −	9.0	
36	6.5	5.5 −	16.5 +
39	7.0	6.0 −	17.0 +
40	5.0	6.0	18.0
44	9.0	8.0 −	
65	7.0	7.0 −	
66	5.0 −	5.0	
68	8.0	9.0 −	18.0 +
70	7.0	7.0 −	16.0
71	6.0 −	5.0	
72	6.0 −	5.0 −	
73	8.0	7.0	

A solution consisting of equal parts of dextrin and sucrose was used. The assimilation limits in nine animals ranged between 5 and 9 gm. per kilo. Thirteen other rabbits received two or more doses of the mixture, but the smallest dose ingested by each still resulted in glycosuria.

The limits of assimilability of sucrose, dextrin, and sucrose + dextrin (1:1) in the same animals are given in Table VI.

An examination of Table VI shows that the assimilation limit of the mixture was less than that of sucrose alone in seven

cases, while the reverse was true in six cases. Dextrin tolerance was, without exception, two or three times greater than that of either pure sucrose or the mixture of sucrose and dextrin. Seven rabbits (Nos. 4, 19, 36, 39, 40, 68, and 70) had an average dextrin tolerance of more than 17.07 gm. per kilo and an average sucrose tolerance of 6.64 gm., while less than 6.64 gm. of the sucrose-dextrin mixture was assimilated perfectly. Thirty-one rabbits had glycosuria after the ingestion of the sucrose-dextrin mixture and of these, twenty-five had sucrose in the urine; six had reducing sugar, and two had both.

Glucose + Dextrin.—A series of experiments was planned to determine whether the addition of dextrin to glucose has any

TABLE VII.

Animal No.	Limits of assimilability per kilo.		
	Glucose.	Dextrin.	Glucose-dextrin (1:1).
	gm.	gm.	gm.
4	14.0	16.0 +	10.0
19	12.0	18.0 +	16.0
21	12.0	18.0	15.0
69	16.0 +	16.0 +	16.0
74	10.0		14.0 +
78	16.0	18.0 +	16.0
79	16.0	16.0 +	16.0 +

effect upon the tolerance of the latter. A solution consisting of equal parts of glucose and dextrin was used. The limit of assimilability was determined in five animals, the doses which just resulted in glycosuria ranging from 10 to 16 gm. per kilo, while eight animals ingested doses ranging from 12 to 16 gm. per kilo without sugar appearing in the urine.

Table VII shows the relative assimilability of glucose, dextrin, and the glucose-dextrin mixture (1:1) in the same animals.

Table VII shows that three of the seven animals had a tolerance for glucose equal to or greater than that for the glucose-dextrin mixture, while in four the reverse was true. The dextrin tolerance was high in all animals, and, in those cases determined, was greater than that for either pure glucose or the glucose-dextrin mixture. It appears, therefore, that dextrin

mixed with glucose in the proportion of 1:1 does not always increase the assimilability of the latter.

History of a Typical Animal.

Rabbit 4.—This animal was a large male weighing at the beginning of the experiments 2,480 gm. After the ingestion of his first dose of sugar, 6.5 gm. per kilo of sucrose, reducing sugar appeared in the urine. Seven and

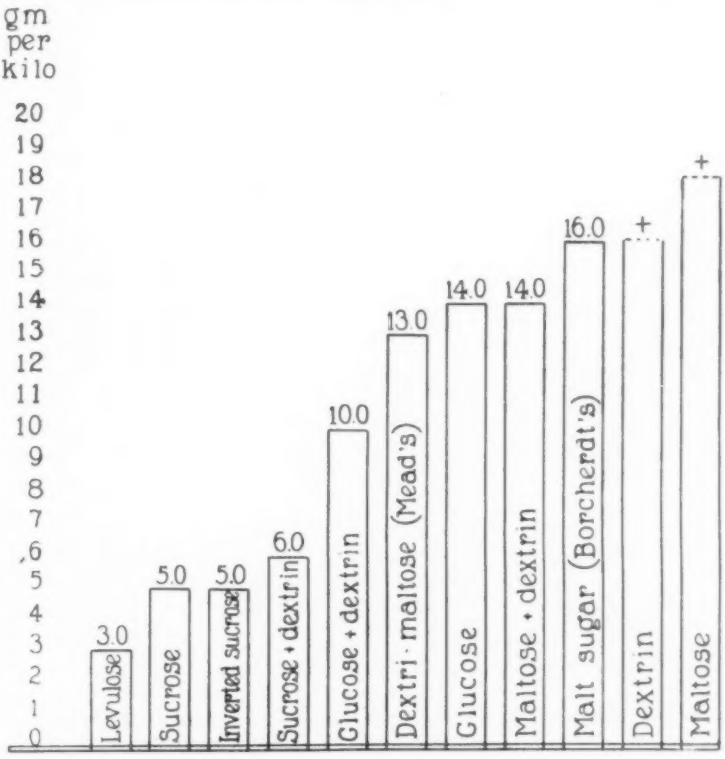

FIG. 1. Graphic representation of limits of assimilability of the various carbohydrates in Rabbit 4.

one-half months later, this animal received his fortieth dose of sugar. During this time he had maintained an approximately constant weight, and appeared to be in a normal condition at all times. The sucrose tolerance in this rabbit was found to be 5 gm. per kilo, reducing sugar *only* appearing in the urine. After he had received a large number of doses of various sugars

during an interval of 3½ months, a second dose of sucrose, 5 gm. per kilo, was administered. As previously found, reducing sugar appeared in the urine. This same dose was repeated 2½ months later, and again reducing sugar was found in the urine. Apparently, repeated doses of sugars at frequent intervals had not affected the sucrose tolerance in this animal.

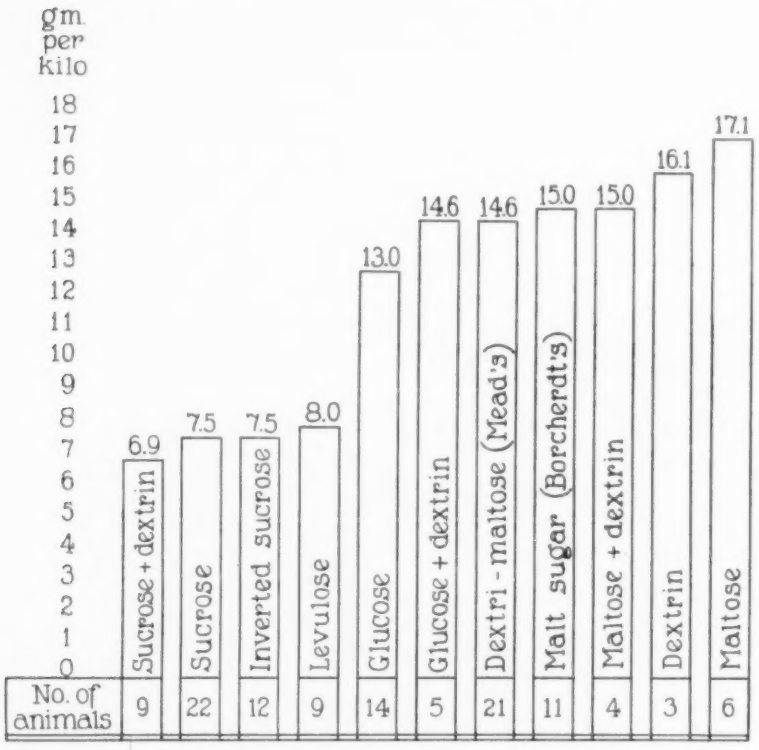

FIG. 2. Summary of observations on urine: graphic representation of the average limits of assimilability of the various carbohydrates.

Compared with other rabbits, No. 4 had a relatively low sucrose tolerance (5 gm. per kilo) and a fairly high dextrin tolerance (16 gm. per kilo). When the two carbohydrates were mixed in the proportion of 1:1, the assimilation limit of the mixture was found to be only 6 gm. per kilo. The assimilation limit of inverted sugar in this animal was the same as that of sucrose, and since reducing sugar invariably appeared in the urine after sucrose ingestion, it is probable that the latter was inverted in the intestinal tract before absorption (Figs. 1 and 2).

From Table VIII it appears that, for the carbohydrates studied, tolerance in rabbits occurred in the following order of increasing assimilability: (1) sucrose; (2) levulose; (3) glucose; (4) maltose and dextrin.

While from the table maltose appears to be more assimilable than dextrin, the reverse is probably true, since the tolerance for the latter was so great that the *limit* of assimilability could not be determined except in a few cases.

TABLE VIII.

Summary of Observations.

Carbohydrates.	Rabbits to which carbohydrates were fed.		Rabbits in which limits of assimilability were determined.		
	Total No.	No. developing diarrhea.	Total No.	Average tolerance.	No. developing diarrhea.
				gm.	
Glucose	18	6	14	13.0	2
Levulose	17	1	9	8.0	0
Inverted sucrose	17	2	12	7.5	2
Sucrose	58	8	22	7.5	1
Maltose	14	1	6	17.1	0
Lactose	4	0	0		
Dextrin	25	0	3	16.1	0
Dextri-maltose	39	6	21	14.6	1
Malt sugar	27	5	11	15.0	0
Maltose (50 per cent) Dextrin (50 " ")	5	0	4	15.0	0
Sucrose (50 " ") Dextrin (50 " ")	22	2	9	6.9	0
Glucose (50 " ") Dextrin (50 " ")	14	2	5	14.6	0

Maltose-dextrin and glucose-dextrin mixtures were highly assimilable, while the sucrose-dextrin mixture was the least assimilable of all the carbohydrates tested.

The limit of assimilability of the maltose-dextrin mixtures averaged, on the whole, less than that of pure maltose or pure dextrin.

Taking all the animals tested into account, the glucose-dextrin mixture was slightly more assimilable than pure glucose and less

assimilable than pure dextrin, but in five animals which received doses of all three preparations the average tolerance for the glucose-dextrin mixture was no greater than that for pure glucose and less than that for pure dextrin.

Diarrhea rarely occurred unless the dose of carbohydrate exceeded the assimilation limit.

STUDIES ON CARBOHYDRATE METABOLISM IN RABBITS.

II. EFFECT OF CARBOHYDRATE FEEDING ON BLOOD SUGAR.*

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In a previous communication by Mendel and Jones[1] feeding experiments with the various carbohydrates ordinarily used in infant feeding were reported. As determined in those experiments, carbohydrate tolerance in rabbits occurred in the following order of increasing assimilability: sucrose, levulose, glucose, dextri-maltose, Borcherdt's malt sugar, maltose, and dextrin. Glucose, maltose, and dextrin were highly assimilable, but when dextrin was added to glucose and maltose in the proportion of 1:1, the mixtures were, in some cases, less assimilable than the pure sugars. A mixture of sucrose and dextrin in equal proportions was the least readily assimilable of all the carbohydrates tested. With these results in mind, two questions arose: first, What relation exists between the sugar in the urine and that in the blood? and second, To what are differences in utilization due?

In order to determine the relation between sugar in the urine and that in the blood, a series of experiments was planned to study the composition of these fluids after the ingestion of com-

* The data in this paper were taken from the dissertation presented by Martha R. Jones for the degree of Doctor of Philosophy, Yale University, 1920. Part of the expense of the investigation was defrayed by a grant from the Russell H. Chittenden Research Fund for Physiological Chemistry.

† Research Fellow (1919–20) of the Boston Branch of the Association of Collegiate Alumnæ.

[1] Mendel, L. B., and Jones, M. R., *J. Biol. Chem.*, 1920, xliii, 491.

parable doses of the various carbohydrates under investigation. Since the limit of assimilability of sucrose is relatively low, comparisons were made on the basis of sucrose tolerance.

Methods Employed.

MacLean's[2] micro method requiring 0.2 cc. of blood was used for the determination of the blood sugar. The accuracy of the method was tested by adding known quantities of glucose to blood samples which had previously been analyzed and determining the total amount of sugar present. Most satisfactory results were obtained. As in all micro methods, extreme care had to be exercised, and all pipettes and flasks kept scrupulously clean. After the addition of the colloidal iron to the coagulated blood mixture the flask should be shaken vigorously for at least 1 minute, a point which is not emphasized by MacLean, but was found to be quite essential. A sort of emulsion is formed which filters quite slowly, but much better duplicates were obtained when this precaution was taken. Two sets of flasks were used, one exclusively for precipitating the proteins and the other for the reduction of the copper solution. The flasks in which the proteins were precipitated (colloidal iron being used) had to be scrubbed after each test, as the iron adhered very closely to the glass and it was found impossible to remove it completely in any other way. One disadvantage of the method is that it requires about 40 minutes for each determination, making frequent tests impossible when observations are being made on both blood and urine. A great advantage is that the method lends itself very well to the determination of sucrose in the blood. An aliquot of the filtrate from the coagulated blood mixture was made acid with ten drops of a concentrated HCl solution and gently boiled for 10 minutes. The flask was stoppered and a long glass tube inserted to prevent evaporation. At the end of 10 minutes the flask was plunged into cold water and the solution titrated with NaOH of such a strength that ten drops exactly neutralized the ten drops of HCl used. One drop of phenolphthalein was used as indicator. Since a small amount of water was lost by evaporation the twenty drops of HCl and NaOH added did not materially change the volume of solution. From this point the procedure was exactly the same as that described by MacLean. The method was tested by adding known quantities of sucrose to the blood filtrate and testing before and after inversion. Satisfactory results were obtained.

Blood samples were taken immediately before and 1 hour after the ingestion of the sugar solution, while urine was collected before and 2 hours after the administration of the sugar. In a few cases the blood was collected at hourly intervals for 3 hours. As reported by numerous investigators, the sugar content invariably reached its maximum within the 1st hour. Each rabbit was weighed and its normal urine collected and tested

[2] MacLean, H., *Biochem. J.*, 1919, xiii, 135.

as previously described. It was then fastened on the animal board and a small incision made in the marginal ear vein, from which the blood was allowed to flow into a small weighing bottle containing a few grains of powdered potassium oxalate. At times some difficulty was experienced in obtaining a good flow of blood, but this was overcome by warming the ear with a lighted electric bulb. As only 0.2 cc. of blood was used for each test, a very small amount was collected. Since blood contains a glycolytic ferment, the sugar tests, in duplicate, were started immediately after the blood was drawn. While the rabbit was still fastened on the board the sugar solution was administered. The animal was then returned to its cage and 1 hour later a second blood sample was taken and tested as above. 2 hours after the ingestion of the sugar the urine was collected and tested for sugar with Benedict's reagent.

The Normal Blood Sugar.

The concentration of sugar in the blood was determined in sixteen normal rabbits, the average being 0.086 per cent. The highest blood sugar content exhibited by any animal was 0.116 per cent, while two had a sugar concentration of only 0.068 per cent. The normal blood sugar of each animal was tested at intervals until three or more determinations had been made, but the variations were found to be negligible and always within the limits of experimental error.

For the rabbit, the following data regarding normal blood sugar values have been collected by Allen.[3]

Investigator.	Blood sugar.
	per cent
Schenck	0.12
Lewandowski	0.09
Andersson and Erlandsen	0.12 to 0.13
Rose	0.15 (Maximum, never up to 0.2.)
Nishi	0.075 to 0.165
Lyttgens and Sandgren	0.222
Wacker	0.21 to 0.27
Schirokauer	0.10 to 0.11

Allen considers the great differences in blood sugar values probably due to the fact that the rabbit is an animal subject to

[3] Allen, F. M., Studies concerning glycosuria and diabetes, Cambridge, 1913.

emotional and traumatic hyperglycemia to an unusual degree, and that the lowest figures are the most trustworthy.

A striking demonstration of extreme emotional hyperglycemia was obtained in one of our own animals. The rabbit was a young male in excellent physical condition but was unaccustomed to close confinement or handling. When placed on the animal board he showed great excitement and his blood sugar had a concentration of 0.187 per cent. After the ingestion of 8 gm. per kilo of glucose the blood sugar content increased to 0.264 per cent, the highest ever obtained, accompanied by extreme glycosuria. A month later this animal's blood sugar was again tested. He still showed some excitement when handled but the blood sugar content was only 0.138 per cent. Recent reports in which improved methods for the determination of blood sugar have been employed tend to show that the normal blood sugar content of the rabbit is much lower than originally thought, in fact, is very near the concentration in man. Allen asserts that it is not improbable that all or most mammals may be found to have a rather constant blood sugar content.

The Relation between Blood Sugar and Urine Sugar after the Ingestion of Certain Carbohydrates.

Glucose.—Eight rabbits received one or more doses of glucose. The relation between the sugar in the blood and that in the urine after the ingestion of glucose is shown in Table I.

Table I shows that hyperglycemia results after the ingestion of glucose, and that the degree, in the same animal, is proportional to the quantity ingested. Only after the concentration of the sugar in the blood reaches a certain degree does it escape into the urine. According to Hamman and Hirschman[4] the blood sugar in man rises rapidly but seldom exceeds 0.15 per cent after the ingestion of glucose. It falls somewhat more slowly to the original level, the whole reaction taking place in less than 2 hours. "If the blood sugar passes 0.18 per cent sugar usually appears in the urine, but sometimes appears at a lower level and at other times fails to appear even though 0.2 per cent is exceeded."

[4] Hamman, L., and Hirschman, I. I., *Johns Hopkins Hosp. Bull.*, 1919, xxx, 306.

The results of the above experiments on rabbits confirm the statement of Hamman and Hirschman. Sugar appeared in the urine only once when the blood sugar content was as low as 0.137 per cent. Five animals had sugar in the urine when the blood sugar concentration was 0.18 per cent or more. In six out of seven cases the doses of glucose ranging from 6 to 9 gm. per kilo produced a hyperglycemia of 0.138 to 0.168 per cent without glucosuria.

Sucrose.—Thirteen rabbits received doses of sucrose ranging from 6 to 8 gm. per kilo. The blood and urine were tested for reducing substances before the ingestion of the sugar, and for

TABLE I.

Animal No.	Glucose per kilo.	Blood sugar.		Sugar in urine.
		Normal.	1 hr. after sugar.	
	gm.	*per cent*	*per cent*	
109	9.0	0.087	0.144	−
109	12.0	0.087	0.186	Trace.
110	6.0	0.09	0.14	−
110	8.0	0.09	0.212	+
111	6.0	0.116	0.168	−
111	8.0	0.116	0.25	++
112	7.5	0.07	0.142	−
113	7.0	0.085	0.137	+
114	7.0	0.08	0.138	−
115	7.0	0.084	0.181	++
116	7.0	0.09	0.165	−
116	10.0	0.09	0.25	++

both reducing substances and sucrose after the ingestion. In twelve of the thirteen rabbits sucrose, as such, appeared in the urine, while in one the assimilation limit was not reached. Five rabbits showed a more or less marked *decrease* in reducing sugar in the blood. After inversion, the *total* sugar content was approximately normal, indicating the presence of sucrose. These rabbits also exhibited sucrosuria to a marked degree. Eight animals showed the same or an increase in reducing substances in the blood after the ingestion of sucrose. In three of these cases, sucrose, as such, was present; in one, no appreciable increase in reduction was noted after inversion; and in four, the presence of sucrose was not determined.

Rabbit 114, which had no increase in blood sugar but marked sucrosuria after the ingestion of 7 gm. per kilo of sucrose, was given a second dose of the sugar (6 gm. per kilo) 5 days after the first. The blood sugar content increased from 0.07 to 0.121 per cent during the 1st hour, only reducing sugar being present. The urine test showed marked reduction, with no increase after inversion. One of two possibilities may have occurred. Either the sucrose was completely inverted in the intestines before absorption, or, if a small portion of the unchanged sugar found its way into the blood stream, an enzyme, evoked by the previous dose of sugar may have inverted it. Neither the dog nor the rabbit has sucrase in its normal serum.[3] Weinland[5] was able by repeated subcutaneous injections of sucrose in dogs to produce an invertin in their serum. In the present investigation the pres-

TABLE II.

Animal No.	Sucrose per kilo.	Blood sugar.			Urine sugar.	
		Normal.	1 hr. after sugar.	After inversion.	Reducing.	Sucrose.
	gm.	*per cent*	*per cent*	*per cent*		
109	7.0	0.086	0.065	(?)	—	—
110	6.0	0.085	0.05	0.081	Trace.	+
111	6.0	0.116	0.055	0.112	+	++
112	7.5	0.07	0.052	0.072	—	+
114	7.0	0.087	0.075	0.089	Trace.	+++

ence of an amylase in rabbit urine after the ingestion of sucrose has been repeatedly demonstrated. No tests for sucrase, however, were made, but it seems reasonable to assume that it might have been present. Rabbit 114 also received 7 gm. per kilo of glucose. The blood sugar content rose to 0.138 per cent without glycosuria. The fact that glycosuria resulted after sucrose ingestion when the concentration of sugar in the blood was only 0.121 per cent and did not result after glucose ingestion when the blood sugar content was 0.138 per cent indicates a relatively low renal threshold for invert sugar.

Table II shows the relation of glycemia to glycosuria in those animals which exhibited a lowered content of reducing sugar in the blood after the ingestion of sucrose.

[5] Weinland, E., *Z. Biol.*, 1906, xlvii, 279.

Table III shows the relation between glycemia and glycosuria in those animals which exhibited an increase in reducing sugar in the blood after the ingestion of sucrose.

Rabbits 103, 107, and 108 showed marked sucrosuria with more or less glucosuria. The sucrose concentration in the blood was also high, while the concentration of reducing sugar was relatively low.

From Tables II to III two distinct phenomena are apparent: first, *hypoglycemia* with *normal total blood sugar*, accompanied by sucrosuria; second, a *relatively low glycemia* with high *total blood sugar* accompanied by both glycosuria and sucrosuria.

TABLE III.

Animal No.	Sucrose per kilo.	Blood sugar.				Urine sugar.	
		Normal.	1 hr. after sugar.	2 hrs. after sugar.	After inversion.	Before inversion.	After inversion.
	gm.	*per cent*	*per cent*	*per cent*	*per cent*		
101	7.5	0.08		0.08		+	++
102	7.0	0.068		0.104		+	++
103	6.0	0.084		0.116	0.187	+	++
104	6.0	0.08		0.08		−	−
107	6.0	0.081	0.137		0.166	+	++
108	7.0	0.067	0.103		0.147	Trace.	++
113	7.0	0.081	0.144		0.144	++	++
115	7.0	0.07	0.125			−	+

Dextri-Maltose.—The blood sugar content was determined in twelve animals after the ingestion of one or more doses of dextri-maltose (Mead's). The relation between the sugar in the blood and that in the urine in these animals is given in Table IV.

Table IV presents, in general, two distinct blood pictures: first, large doses of sugar resulting in relatively low hyperglycemia accompanied by glycosuria; second, large doses of sugar with little or no increase in blood sugar and no sugar in the urine. In all cases except two (Rabbits 104 and 112) an increase in dose resulted in an increase in blood sugar. Rabbits 105, 108, 111, and 116 ingested 14, 7, 6, and 10 gm. per kilo, respectively, without an appreciable increase in blood sugar or glycosuria resulting. Rabbits 103, 104, and 116 received 14 gm. per kilo each with only a slight increase in blood sugar although there was a small

amount of sugar in the urine. It is possible that the sugar was so slowly absorbed in these cases that the maximum blood sugar value was not attained during the 1st hour.

Dextrin.—Owing to our inability to obtain a sufficient amount of purified dextrin, only three doses were given. Rabbits 109, 112, and 113 received 12, 10, and 7 gm. per kilo, respectively, of a so called chemically pure dextrin, but a different product from that used in previous experiments. The highest blood sugar value attained was 0.145 per cent. Glycosuria resulted in two cases.

TABLE IV.

Animal No.	Dextri-maltose per kilo.	Blood sugar.		Sugar in urine.
		Normal.	1 hr. after sugar.	
	gm.	*per cent*	*per cent*	
101	7.5	(?)	0.093	–
103	14.0	0.09	0.116	Trace.
104	14.0	0.087	0.106	"
104	12.0	0.087	0.13	–
105	14.0	0.112	0.121	–
106	15.0	0.085	0.202	++
106	10.0	0.085	0.108	–
107	10.0	0.082	0.162	–
107	6.0	0.082	0.142	–
108	10.0	0.062	0.15	–
108	7.0	0.062	0.075	–
110	6.0	0.081	0.103	+
111	6.0	0.116	0.127	–
112	10.0	0.07	0.125	–
112	7.5	0.07	0.121	–
114	10.0	0.087	0.105	–
116	14.0	0.09	0.133	+
116	10.0	0.09	0.091	–

Comparing the blood sugar values after the ingestion of glucose and dextri-maltose it appears that comparable and even larger doses of the latter result in relatively lower concentrations of blood sugar (Table V). In addition to the above carbohydrates, Rabbit 109 received 12 gm. per kilo of pure maltose, the blood sugar content rising to 0.16 per cent 1 hour after the ingestion of the sugar. It seems that the glucose threshold value in this animal was approximately 0.144 per cent, since a trace of sugar

appeared in the urine when the blood sugar attained that concentration after ingestion of 9 gm. per kilo of glucose, and positive tests resulted when glycemia increased to 0.145, 0.16, and 0.186 per cent after the ingestion of 12 gm. per kilo of dextrin, maltose, and glucose respectively. These relative values lend credence to the theory that polysaccharides are more slowly digested and

TABLE V.

Comparison of Blood Sugar Values in Selected Animals.

Animal No.	Normal blood sugar.	Sucrose.			Glucose.		Dextri-maltose.		Dextrin.	
		Amount ingested.	Blood sugar before inversion.	Blood sugar after inversion.	Amount ingested.	Blood sugar.	Amount ingested.	Blood sugar.	Amount ingested.	Blood sugar.
	per cent	*gm.*	*per cent*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*
109	0.086	7.0	0.065		9	0.144	12.0	0.137	12	0.145
					12	0.186				
110	0.085	6.0	0.05	0.081	6	0.14	6.0	0.103		
					8	0.212	10.0	0.140		
111	0.116	6.0	0.055	0.112	6	0.168	6.0	0.127		
					8	0.25	11.0	0.17		
112	0.07	7.5	0.052	0.072	7.5	0.142	7.5	0.121	10	0.143
							10.0	0.125		
113	0.081	7.0	0.144	0.144	7	0.138			7	0.137
114	0.087	6.0	0.121	0.131	7	0.138	10.0	0.105		
		7.0	0.075	0.089						
116	0.09				7	0.165	10.0	0.091		
					10	0.25	14.0	0.133		

absorbed than monosaccharides, and that upon the rate of absorption depends the ability of the liver and tissues to utilize the excess. Two rabbits (Nos. 112 and 109) showed no higher blood sugar after the ingestion of 10 and 12 gm. per kilo of dextrin, respectively, than after the ingestion of 7.5 and 9 gm. of glucose, which indicates slower absorption of the former. Rabbit 113 had a blood sugar content of 0.137 per cent accompanied by

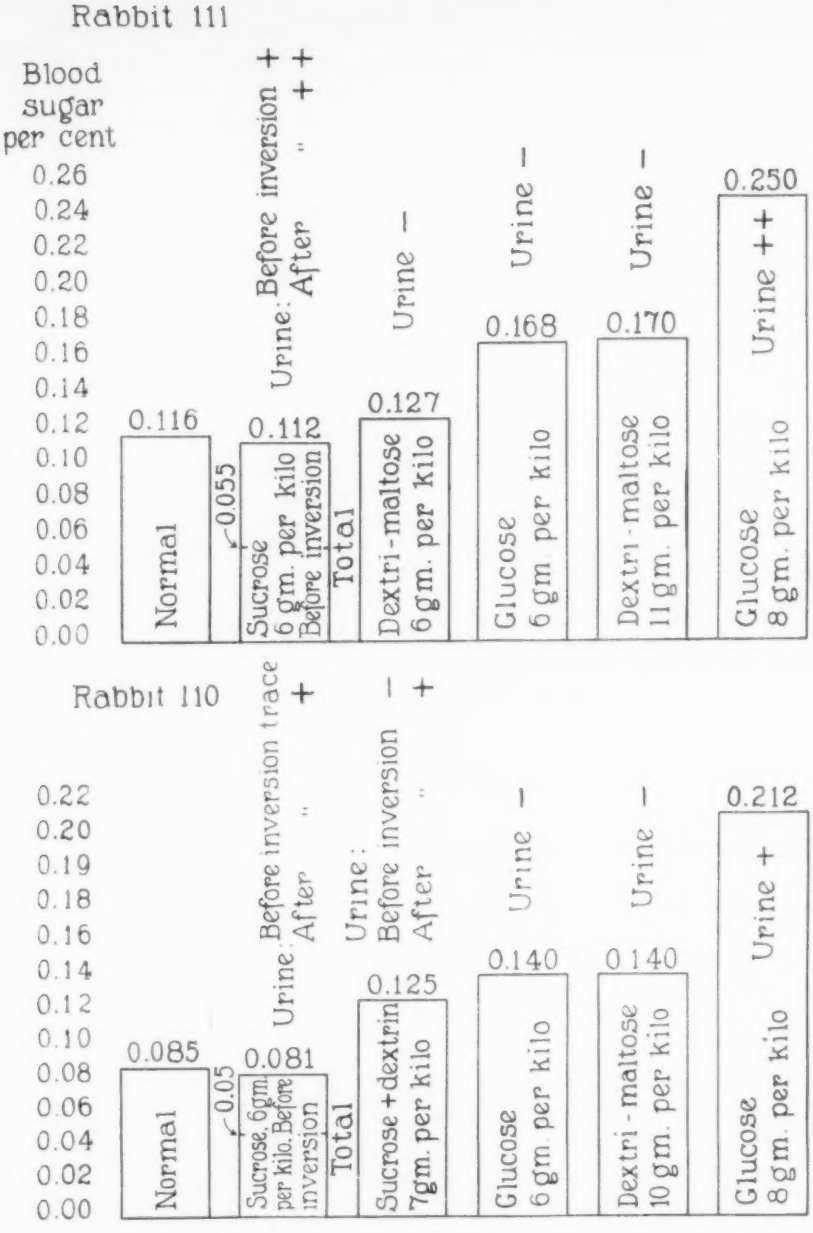

FIG. 1. Graphic representation of blood sugar content of certain individual animals.

glycosuria after the ingestion of glucose, while a comparable dose of dextrin resulted in the same concentration of sugar in the blood but without glycosuria. This also can be explained on the ground of more rapid initial absorption of glucose, its maximum value probably having been attained before the expiration of the hour.

In the few experiments in which two or more doses of glucose were given to the same animal, the sugar concentration in the blood invariably increased with the size of the dose. Sucrose in the same dosage as glucose, with one exception, resulted in no increase in total blood sugar, the reducing sugar being below normal (Fig. 1).

DISCUSSION.

When sugar is ingested orally its passage into the urine may be prevented in three ways: first, by the liver which converts it into glycogen; second, by the tissues which metabolize it; and third, by the kidneys which fail to excrete it unless the concentration in the blood exceeds a certain level.

Bernard established the theory that the kidneys were essentially like a dam to a reservoir. The blood sugar could stand at a certain level without loss, but if this level for any reason should be raised the excess flowed over the dam until the original height was restored. This conception is still common in the literature, but recent investigations show that in the normal organism the power to utilize sugar is practically unlimited. An increase in dose means an increase in utilization, and the quantity of sugar excreted is invariably an exceeding small proportion of the sugar ingested. In this respect the normal organism differs from that of the total diabetic in whom the kidneys regulate the height of the blood sugar by allowing the excess above a certain level to escape quantitatively in the urine.

In a recent paper by Hamburger[6] observations on the permeability of the kidney to various sugars were reported. From these experiments it appears that the kidney is perfectly permeable to the three disaccharides as well as to fructose and levorotatory glucose. In perfusion liquids containing mixtures of

[6] Hamburger, H. J., *Brit. Med. J.*, 1919, i, 267.

glucose and levulose, also glucose and lactose, both the levulose and lactose passed through the glomeruli quantitatively, while the retention of glucose was not altered. According to Hamburger: "The two sugars are simply separated from each other as through a filter . . . ." Apparently, the glomerular epithelium has the power of retaining free glucose and distinguishing it from other sugars, a power which is governed by the influence of the chemical composition of the perfusion liquid upon the glomerular membrane.

On the basis of this hypothesis most of the phenomena observed in the present research can be interpreted. Since the kidney appears to be perfectly permeable to sucrose, levulose, and maltose, once they enter the blood stream they are excreted. Since, however, a maltase occurs in the blood, any maltose which escapes into the circulation is rapidly converted into glucose and none escapes into the urine until the concentration in the blood exceeds a certain limit. On the other hand, sucrose entering the circulation, as such, is rapidy excreted, since the normal blood serum contains no sucrase. The addition of dextrin to sucrose facilitated the passage of the latter into the blood stream, and hence even relatively small doses gave rise to glycosuria. The fact that sucrose was nearly always present in the urine after the ingestion of the mixture makes this seem more probable. That levulose is more assimilable than sucrose can be explained on the ground that less of it enters the systemic circulation, owing to its ready conversion into glycogen by the liver. This assumption is supported by the fact that after parenteral injections of levulose relatively small quantities are utilized by the tissues. Since polysaccharides have to undergo hydrolysis in the intestinal tract before absorption, it is reasonable to assume that absorption takes place more slowly after the ingestion of starch and dextrin than after the ingestion of glucose and maltose. Why a glucose-dextrin mixture in some cases had no greater limit of assimilability than pure glucose we are unable to say, unless in the presence of dextrin the preformed glucose undergoes more rapid absorption.

Kidney Function Test.

Since the kidney is an important factor in preventing the escape of sugar into the urine, it seemed desirable to test what effect, if any, large doses of sugar have upon kidney function, and for this purpose the phenolsulfonephthalein test was employed.

0.5 cc. of a phenolsulfonephthalein solution containing 3 mg. of the dye was diluted to 1 cc. with distilled water and injected subcutaneously into the rabbit immediately after the ingestion of 50 cc. of distilled water or sugar solution. The urine was collected in some cases after 2 hours and in others after 3 hours. Two or three drops of concentrated NaOH solution were added to the urine to develop the color and the volume was made up to 500 cc. with distilled water. The percentage of the dye in the urine was determined by matching the color in a Hellige colorimeter against a standard solution containing 3 mg. of phenolsulfonephthalein, 15 cc. of normal urine, and two or three drops of concentrated NaOH made up to a volume of 500 cc. Solutions of sucrose, maltose, dextrin, dextri-maltose, maltose + dextrin, and glucose + dextrin were fed, the excretion of the dye being well within the normal limits in all cases both in the 2 and 3 hour periods. Apparently, large doses of these carbohydrates have no appreciable effect upon kidney function as determined by this test.

SUMMARY.

Comparable doses of carbohydrates gave rise to hyperglycemia in the following order of increasing blood sugar values: dextrin, dextri-maltose, and glucose.

Sucrose ingested in doses sufficient to cause glycosuria presented three distinct phenomena: (1) *hypo*glycemia with normal total blood sugar content accompanied by sucrosuria; (2) a relatively low glycemia with high total blood sugar value accompanied by both glycosuria and sucrosuria; (3) *hyper*glycemia accompanied by glycosuria.

In the few determinations made, maximum blood sugar values were attained 1 hour after the ingestion of glucose and dextri-maltose, an increase in dose resulting, in general, in an increase in blood sugar content.

STUDIES ON PROTEINOGENOUS AMINES.

VI. THE PREPARATION OF HISTIDINE FROM BLOOD CORPUSCLE PASTE.

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Since Fränkel[1] published his method for the preparation of histidine, small quantities of this substance have been obtainable without difficulty. The details given by Fränkel are rather incomplete. Most laboratories have found it necessary, therefore, to supply the exact details in order that their students could prepare this amino-acid successfully. One very good unpublished modification with which we are familiar is that devised by Dr. F. C. Koch of the Department of Physiological Chemistry at the University of Chicago. Jones,[2] who was formerly one of Koch's students, has recently published a method for preparing histidine that is essentially that of Koch.

Anyone who has prepared histidine repeatedly by one of these processes cannot fail to have been struck by the uncertainty of the results. The yield of histidine dichloride may vary between the wide limits of 10 to 25 gm. from 2 liters of the same corpuscle paste. Frequently the presence of impurities in the crude histidine dichloride makes it difficult to obtain a good yield of the pure substance. The most serious causes of difficulty seem to be the following.

1. The precipitation of humin and ferric hydroxide by means of sodium carbonate is very unreliable. Even an experienced chemist may waste considerable time before the correct condition for complete precipitation is obtained. By using lime in place of sodium carbonate this difficulty can be removed entirely.

[1] Fränkel, S., *Monatsh. Chem.*, 1903, xxiv, 230.

[2] Jones, H. M., *J. Biol. Chem.*, 1918, xxxiii, 429.

2. The mercury salt of histidine has usually been prepared by adding sodium carbonate and mercuric chloride alternately until precipitation has ceased. We have found that *the precipitation is always incomplete unless an excess of mercuric chloride is present from the start*; that it is necessary to use far more mercuric chloride than has usually been employed; and that by using exact weights of mercuric chloride and sodium carbonate a uniformly complete precipitation can always be obtained.

3. The final purification of the histidine dichloride by the previously described methods has been subject to considerable loss both of time and material. By recrystallizing the crude product from aqueous alcohol under exactly defined conditions, a very good yield of a perfectly pure final product is obtained. The process to be described has given an average yield of 15 gm. of pure histidine dichloride from 500 cc. of blood corpuscle paste, which is about four times the quantity that has usually been obtained.

EXPERIMENTAL.

1. Hydrolysis.—Fresh blood corpuscle paste,[3] 500 cc., is mixed with 1,000 cc. of 37 per cent hydrochloric acid in a *weighed* 3,000 cc. long necked, round bottomed Pyrex flask and hydrolyzed by boiling, under a reflux condenser, for 30 hours over an electrically heated asbestos bath.

2. Removal of the Hydrochloric Acid.—The hydrochloric acid is removed by distillation *in vacuo* at 60° from the same flask. The residue is finally dried at 100° for 2 hours to remove the water and acid as completely as possible. The flask is weighed and the weight of the residue—usually about 350 gm.—is obtained by difference. This weight is very important because the quantities of mercuric chloride and sodium carbonate used later in the preparation depend upon it. This will be referred to later as Residue 2.

3. Removal of Ammonia.—Residue 2 is dissolved in 1,000 cc. of water. The solution is treated with commercial finishing lime until the reddish brown precipitate formed at first assumes a homogeneous clay color due to the presence of undissolved lime.

[3] The blood corpuscle paste was obtained from Armour and Company, Chicago, Ill.

The saturation with lime is necessary to insure a complete precipitation of the ferric hydroxide and humin. The mixture is treated with 500 cc. of 95 per cent alcohol and subjected to a distillation *in vacuo* at 40° until about 800 cc. of distillate have been collected. This removes the ammonia completely.

4. Removal of Humin and Ferric Hydroxide.—The mixture is filtered, on a 6 inch Buchner funnel, from humin, ferric hydoxide, and excess lime, the precipitate being carefully washed with a large volume—2,000 cc.—of a hot saturated aqueous solution of lime. The clear amber-colored liquid, which contains all the amino-acids as lime salts, is always free from iron when enough lime has been added.

5. Isolation of Tyrosine and Leucine.—The alkaline filtrate obtained under Section 4 is diluted to 4,000 cc., heated on the water bath, and treated with 350 gm.[4] of solid anhydrous sodium carbonate. The resulting mixture is agitated until all the sodium carbonate has passed into solution. This precipitates the calcium as calcium carbonate. The mixture is filtered promptly on a 6 inch Buchner funnel and the precipitate is washed with 1,000 cc. of hot water. The filtrate, which should be free from calcium, is transferred to a 6 liter flask, cooled under the tap, and treated with 37 per cent hydrochloric acid *until the liquid reacts neutral to litmus paper.* Glacial acetic acid is then added until effervescence ceases. The solution is subjected to a distillation *in vacuo* at 50–60° until its volume has been reduced to about 800 cc. Sodium chloride crystallizes out toward the end of the distillation together with small quantities of tyrosine and leucine. The mixture is placed in the ice chest for 4 days which separates the tyrosine almost completely, and considerable leucine. It is then filtered on a 5 inch Buchner funnel, the precipitate being washed with 200 cc. of ice-cooled water.

The precipitate contains about 50 gm. of leucine and 1.5 gm. of tyrosine together with a large quantity of sodium chloride. The

[4] The weights of mercuric chloride and sodium carbonate given here are correct only when the weight of Residue 2 (p. 522) is 350 gm. The weight of this residue is, of course, dependent upon the quality of the blood corpuscle paste. In case Residue 2 does not weigh approximately 350 gm., the quantities of $HgCl_2$ and Na_2CO_3 to be used can be obtained by proportion.

separation of the leucine from the tyrosine and the further purification of these two amino-acids can be most easily accomplished by the method of Habermann and Ehrenfeld.[5]

The filtrate, which contains the histidine, is diluted to exactly 2,000 cc. It will be referred to as Filtrate 5.

6. Precipitation of the Mercury Compound of Histidine.—The filtrate from the tyrosine and leucine—Filtrate 5, volume 2,000 cc.—is divided into four equal parts.[6] Each 500 cc. portion is transferred to a 6,000 cc. flask and diluted with 1,500 cc. of water. The further discussion will be limited to *one* of these portions. The other three portions are treated in a manner identical to that which will be described.

Solid mercuric chloride—350 gm.,[1] *four times the weight of Residue 2 which is present in this portion*—is added to the acid liquid. The mixture is heated on the water bath until the sublimate has passed into solution. A small quantity of a gray to brown flocculent precipitate is always present. This can be more advantageously removed later. The liquid is cooled. The mercury salt remains in solution. A solution of sodium carbonate containing 70 gm.[7] of anhydrous salt dissolved in 3,000 cc. of water, is carefully added to the above liquid. This precipitates the mercury salt of histidine in the form of a flocculent white solid that settles readily leaving a clear supernatant liquid. A test portion of this liquid should give no immediate precipitate when it is treated with a sodium carbonate solution. The clear supernatant liquid is removed as completely as possible by means of a glass siphon. Distilled water—about 4,000 cc.—is poured into the flask, the mixture thoroughly agitated, and the precipitate allowed to settle. The supernatant liquid is siphoned off as before. The mercury salt is washed seven times in this manner.

7. Isolation and Purification of Histidine Dichloride.—The four batches of mercury salt obtained under Section 6 are combined in a 6 liter flask. Hydrochloric acid—37 per cent—is then added until

[5] Habermann, J., and Ehrenfeld, K., *Z. physiol. Chem.*, 1902-03, xxxvii, 18.

[6] This division of material was necessary because of the limited size of the laboratory glassware. Then too, the final volume of the entire portion would be 20 liters which is too large a quantity to be handled easily.

[7] In general use 20 gm. of anhydrous sodium carbonate for every 100 gm. of mercuric chloride.

all the white solid has passed into solution. A small quantity of a gray to brown flocculent precipitate is always left that will not dissolve in hydrochloric acid. The mixture is filtered through a large folded filter paper into a 2 gallon bottle. The pale yellow filtrate is saturated with hydrogen sulfide under pressure which removes the mercury completely. The resulting mixture is filtered on a 6 inch Buchner funnel, and the clear *colorless* filtrate is subjected to a vacuum distillation at 60° in a weighed flask. The resulting pale yellow, exceedingly stiff gum is freed from water and hydrochloric acid as completely as possible by heating *in vacuo* at 80° for 2 hours. The gum so obtained—60 to 65 gm.—is dissolved in 60 cc. of 37 per cent HCl by heating on the water bath. The resulting pale brown solution should be free from crystalline matter[8] and will usually remain clear for days if it is kept at room temperature. A few crystals of histidine dichloride are added and the sides of the vessel scratched with a glass rod. The crystallization of histidine dichloride is immediate and so copious that the mass sets to the consistency of paste in the course of 10 minutes. The mixture is placed in an ice bath where it is kept for 2 days to complete the precipitation of histidine dichloride; it is then filtered on a 3 inch Buchner funnel.[9] The white granular powder is washed first with about 50 cc. of cold 37 per cent HCl and then with a cold mixture containing 20 cc. of 37 per cent HCl and 20 cc. of alcohol. After drying at 100° for 10 hours, this solid, which is nearly pure histidine dichloride, weighs from 17 to 19 gm. The following concrete example illustrates the further purification of this product.

Of the crude histidine dichloride, 30 gm. are dissolved in 20 cc. of hot water. Hot 95 per cent alcohol—200 cc.—is added to the above aqueous solution. The liquid is heated on the water bath until the alcohol boils,[10] when it is filtered through a small

[8] A slight crystalline residue is usually sodium chloride which indicates that the mercury compound of histidine was not washed sufficiently. This inorganic matter can be more advantageously removed later.

[9] It is best to use two thicknesses of a fairly hard but not too retentive a grade of filter paper in this case.

[10] When the entire process has been properly conducted, the aqueous alcoholic solution will contain no crystalline residue. If such a residue is present it consists of inorganic salts. These are then removed by the filtration that is carried out in any case to remove shreds of filter paper and bits of broken glass.

folded filter into a 300 cc. Pyrex flask. The clear, nearly colorless filtrate slowly deposits large colorless plates of histidine dichloride. To hasten the crystallization these first crystals are titrated with a glass rod. The mixture is allowed to crystallize for 24 hours in the ice chest after which it is filtered with suction on a platinum cone, the crystals being washed with 50 cc. of cold 95 per cent alcohol. The pure white product so obtained, after drying *in vacuo* over sulfuric acid for 48 hours, is 100 per cent pure histidine dichloride.[11] The first crop weighs from 21 to 22 gm. A second crop—about 6 gm.—of equally pure material can be obtained from the mother liquor from Crop 1 by repeating the above recrystallization with the same proportions of water and alcohol.

[11] For a typical analysis see Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 502.

STUDIES ON PROTEINOGENOUS AMINES.

VII. THE QUANTITATIVE COLORIMETRIC ESTIMATION OF HISTIDINE IN PROTEIN AND PROTEIN-CONTAINING MATTER.

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INTRODUCTION.

The Relation of Imidazole Derivatives to Certain Biochemical Problems.

The discovery of the imidazole nucleus in histidine gave rise to considerable speculation as to the possible mode of formation of this heterocyclic ring in living matter. The first suggestion as to its mode of formation was obtained from the then well known discovery by Debus[1] that glyoxal condenses with ammonia and formaldehyde to give imidazole. Windaus and Knoop[2] proved in 1905 that methyl imidazole is formed when glucose is allowed to stand for some time in the presence of zinc ammonium hydroxide. The work of these authors leaves little doubt that the methyl imidazole is formed by a condensation of methyl glyoxal and formaldehyde with ammonia, the two aldehydes having been previously formed from the glucose by the action of the ammonia. It is hardly necessary to say that the above condensations, occurring as they do only in the presence of a high concentration of ammonia, can scarcely be claimed to approach the conditions as we find them in living matter. The synthesis of imidazole derivatives by protoplasm may, nevertheless, involve building stones similar to those employed in the laboratory.

[1] Debus, H., *Ann. Chem.*, 1858, cvii, 204.

[2] Windaus, A., and Knoop, F., *Ber. chem. Ges.*, 1905, xxxviii, 1166.

Although histidine seems to be more abundant, in living matter, than any other imidazole derivative, there are others that may prove to be quite as important. Among these we might mention carnosine (β-alanyl-histidine[3]); histamine[4] (β-imidazolyl-ethylamine), a substance that has recently attracted considerable attention because of its powerful physiological activity; the purines, that can be considered as condensation products of the imidazole with the pyrimidine ring; the hydantoins; the closely related glycocyamidines; and creatinine. The last three of the above compounds, although they do not contain a typical imidazole nucleus, have a nuclear cyclic structure like that of the imidazoles. It is possible that a genetic relation exists between all these imidazoles; but this problem as well as the fate of the imidazoles in the animal organism, their relation to the secretagogues and the physiological action of organ extracts, the rôle they play in the diazo reaction of the urine, and many other related questions, could not be successfully approached as long as we were not in possession of an exact, simple, and rapid method for the estimation of imidazole derivatives.

The Method in Brief.

Histidine has been determined in the past by the original method of Kossel and Kutscher,[5] by one of the numerous modifications of this method,[6] and by the group method of Van Slyke.[7] The present method, although it may be no more accurate than the best of those referred to above, has the advantages of being simple, rapid, and direct. The principles involved can be summarized briefly as follows:

[3] Baumann, L., and Ingvaldsen, T., *J. Biol. Chem.*, 1918, xxxv, 263.

[4] See Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 539, for a discussion of previous work on histamine; and Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1920, xliii, 543, for a method of estimation of this substance in complex mixtures.

[5] Kossel, A., and Kutscher, F., *Z. physiol. Chem.*, 1901, xxxi, 165.

[6] Kossel, A., and Patten, A. J., *Z. physiol. Chem.*, 1903, xxxviii, 39. Steudel, H., *Z. physiol. Chem.*, 1903, xxxvii, 219. Kossel, A., and Pringle, H., *Z. physiol. Chem.*, 1906, xlix, 318. Osborne, T. B., Leavenworth, C. S., and Brautlecht, C. A., *Am. J. Physiol.*, 1908, xxiii, 180.

[7] Van Slyke, D. D., *J. Biol. Chem.*, 1911–12, x. 15.

The material is hydrolyzed by boiling with hydrochloric acid. The acid and the volatile phenols, if such are present, are removed by distillation *in vacuo*, after which ammonia and humin are removed by treatment with lime. The material is then divided into two fractions by means of phosphotungstic acid. The phosphotungstate *precipitate* contains the *histidine* together with arginine, lysine, and cystine. Of these four amino-acids, histidine is the only one that gives an orange-red color with an alkaline solution of *p*-phenyldiazoniumsulfonate. Tyrosine, which is, to the best of our present knowledge, the only substance that is normally present in non-putrid, protein-containing matter that could interfere with the colorimetric estimation of histidine, is *not* precipitated by phosphotungstic acid from a dilute solution. The phosphotungstate precipitate is treated with water and sufficient 3 N NaOH to give a clear solution. Histidine is then estimated colorimetrically in this liquid, using the method previously described by us,[8] which is a modification of the familiar qualitative Ehrlich diazo reaction.

Histamine and tyramine, both of which give a pink color with *p*-phenyldiazoniumsulfonate, are also precipitated by phosphotungstic acid. If present they would be estimated as histidine. These amines are, however, never present in non-putrid, protein-containing matter in sufficient quantity to have the slightest effect upon the results.

The method depends, therefore, not upon the actual isolation of histidine, which is time-consuming and subject to the possibility of considerable error, but upon the quantitative determination of the imidazole ring.

Detailed Description of the Method.

The general mode of procedure can be summarized in the following six steps.

1. Hydrolysis.—The protein—1 to 3 gm.—is mixed with 60 cc. of 20 per cent hydrochloric acid in a 400 cc. long necked, round bottomed flask and hydrolyzed by boiling for 28 hours over an electrically heated sand or asbestos bath.

2. Removal of the Hydrochloric Acid and Volatile Phenols.—The hydrochloric acid and the volatile phenols are removed by

[8] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 497.

distillation *in vacuo* at 60° from the same flask. The residue is finally dried *in vacuo* at 80° for 1 hour to remove the last traces of the free hydrochloric acid.

3. Removal of Ammonia.—The residue is dissolved in 100 cc. of water and the solution treated with an excess of lime and 50 cc. of 95 per cent alcohol. The ammonia, alcohol, and some of the water are then removed by distillation *in vacuo* at 40° (again from the same flask).

4. Removal of Humin.—The mixture is filtered, on a Buchner funnel, from humin and excess lime, the precipitate being carefully washed with a large volume of *hot* water until the washings give a negative Pauly reaction.

5. Preparation of the Phosphotungstates.—The alkaline filtrate is acidified by adding a slight excess of hydrochloric acid. The resulting clear, amber-colored liquid is then evaporated to dryness on the water bath in a glass dish. The residue is transferred to a 300 cc. Pyrex Florence flask with a solution of 18 cc. of 37 per cent hydrochloric acid in enough water to give a total volume of 100 cc. The resulting solution is heated on the water bath. To it are then added 100 cc. of a hot 15 per cent aqueous solution of phosphotungstic acid. The resulting mixture is digested for ½ hour on the water bath. It is then allowed to cool slowly to room temperature, after which it is placed in the ice chest for 48 hours[9] and finally cooled in an ice bath for 24 hours. Phosphotungstates have usually been precipitated at room temperatures. Histidine phosphotungstate is far more soluble at 20° than it is at 0° (see experimental part). By conducting the precipitation at 0°, 0.00571 gm. of histidine remains in solution in 200 cc. of precipitation liquid; a definite quantity that can be accurately corrected for. This quantity will be referred to later as the *solubility correction blank.*

6. The Colorimetric Determination of Histidine.—The ice-cold mixture obtained under Section 5 is filtered with the aid of suction, two thicknesses of filter paper and a platinum cone being used in preference to the Buchner funnel process described by Van Slyke.[7] The precipitate is washed freely with an ice-cold liquid containing 18 cc. of 37 per cent hydrochloric acid and 15 gm. of phosphotungstic acid in a total aqueous volume of

[9] Up to this point the method is essentially that of Van Slyke.[7]

200 cc., *the liquid having been previously saturated with histidine phosphotungstate.* Since the wash liquid is already saturated with histidine phosphotungstate it can dissolve none of this substance from the precipitate. No correction, therefore, need be made for the solubility of the precipitate in the wash fluid.

The well washed precipitate finally obtained is transferred, with the paper, to a 1,000 cc. lipped beaker. Enough of a 3 N sodium hydroxide solution is then added to dissolve the precipitate, a large excess of alkali being carefully avoided. The mixture is filtered through a folded filter paper into a 1,000 cc. volumetric flask, and the beaker and filter pulp are thoroughly washed out with distilled water. The clear, pale yellow filtrate is finally diluted to 1,000 cc. This solution will be referred to later as the *test liquid.* The colorimetric determinations are performed on this liquid according to the method previously described by us.[8] The sodium phosphotungstate, which is always present in this liquid, does not interfere with the color production.

The following results have been obtained by using this method.

Histidine Content.

	Colorimetric.	Other methods.	
	per cent	*per cent*	
		2.09	Van Slyke, using the method of Osborne, Leavenworth, and Brautlecht with preliminary phosphotungstic acid precipitation at room temperature (Van Slyke, D.D., *J. Biol. Chem.*, 1913–14, xvi, 533).
Casein.	2.84 first determination.	2.30	Van Slyke, direct precipitation with $AgNO_3$ and baryta (Van Slyke, D.D., *J. Biol. Chem.*, 1913–14, xvi, 535).
	2.84 second determination.	3.37	Van Slyke by group method (Van Slyke, D.D., *J. Biol. Chem.*, 1913–14, xvi, 537).
		2.59	(Abderhalden, E., *Z. physiol. Chem.*, 1905, xliv, 23.)
Edestin.	3.04	2.40	(Osborne, T. B., and Liddle, L. M., *Am. J. Physiol.*, 1910, xxvi, 295.)

Hemoglobins.

	Colorimetric.	Other methods.
	per cent	*per cent*
Horse (1)	8.9	10.5 (Abderhalden, E., *Z. physiol. Chem.*, 1902-03, xxxvii, 492.)
(2)	8.65	
Cat.	8.5	
	8.55	
Sheep.	8.8	
Ox.	7.93	8.15 (Van Slyke, D. D., *J. Biol. Chem.* 1911-12, x, 52.)

Whole blood (normal human).
1.5731 gm. of histidine per 100 cc. of hydrolyzed blood.
Blood serum (normal human).
0.2338 gm. of histidine per 100 cc. of hydrolyzed blood serum.

EXPERIMENTAL.

Sodium Phosphotungstate Does Not Interfere with the Color Produced When Histidine Reacts with p-Phenyldiazonium Sulfonate.

Phosphotungstic acid—15 gm.—was dissolved in 85 cc. of water contained in a 100 cc. volumetric flask. Histidine dichloride solution—*2 cc. of a 1 per cent solution*—was then added together with enough water to give a total volume of 100 cc. The liquid remained clear. Of this solution, 5 cc. were transferred to a 25 cc. volumetric flask, neutralized with a sodium hydroxide solution, and diluted to 25 cc. Of this diluted solution

0.50 cc. had a color value equivalent to 10.0 mm. (CR-MO)[10]
1.00 " " " " " " " 19.5 " (CR-MO)

For the 0.50 cc. portion this is equal to 0.02 gm. of histidine dichloride for the entire original liquid, 100 per cent of the amount actually introduced.

For the 1.00 cc. portion this is equal to 0.0195 gm. of histidine dichloride for the entire original liquid, 97.5 per cent of the amount actually introduced.

[10] The details of the colorimetric method and an explanation of the symbols employed here have been described.[8] This paper also contains tables by means of which colorimetric readings can be readily transformed into gm. of histidine, histamine, etc.

These results show that sodium phosphotungstate, in concentrations as high as 15 per cent, does not interfere with the accuracy of the colorimetric determination of histidine.

The Solubility of Histidine Phosphotungstate.

Experiment A.—Histidine dichloride—0.1000 gm.—was mixed with 82 cc. of water and 18 cc. of 37 per cent hydrochloric acid in a 300 cc. Pyrex flask. The solution was then treated with 100 cc. of a 30 per cent aqueous solution of phosphotungstic acid. The clear liquid was placed in the ice chest. At the end of 24 hours a precipitate of histidine phosphotungstate had formed and collected on the bottom of the flask. To ascertain how much histidine remained in solution 1 cc. of the clear supernatant liquid was transferred to a 10 cc. precision cylinder, neutralized to litmus paper with sodium hydroxide, and diluted to 10 cc. Of this solution

0.50 cc. had a color value equivalent to 7.0 mm. (CR–MO)
1.00 " " " " " " " 14.1 " (CR–MO)

This, by table,[10] shows that the equivalent of 0.056 gm. of histidine dichloride was still in solution in 200 cc. of liquid at the end of 24 hours.

The precipitate was triturated with a glass rod, the mixture then being allowed to stand in the ice chest for 43 more hours. At the end of this time 1 cc. of the clear supernatant liquid was removed, neutralized, and diluted to 5 cc. Of this solution

0.50 cc. had a color value equivalent to 2.5 mm. (CR–MO)
1.00 " " " " " " " 4.8 " (CR–MO)

This, by table, shows that the 200 cc. of precipitation liquid still contained the equivalent of 0.010 gm. of histidine dichloride which is equal to 0.0068 gm. of histidine base. Longer standing or cooling to 0° in an ice bath produced no change in this value.

To ascertain the effect of rise in temperature upon the solubility of histidine phosphotungstate, the mixture was allowed to stand on the laboratory table for 24 hours at a room temperature that varied from 27–32°. Then 1 cc. of the clear supernatant

liquid was removed, neutralized, and diluted to 5 cc. as before. Of this solution

0.50 cc. had a color value equivalent to 13.3 mm. (CR–MO)

This, by table, is equivalent to 0.0532 gm. of histidine dichloride—0.0364 gm. of histidine base—for the entire 200 cc. of precipitation liquid.

From this experiment it is clear that histidine phosphotungstate precipitates slowly and that its solubility is markedly influenced by changes in temperature.

Experiment B.—A precipitation was now conducted under the conditions specified by Van Slyke.[7] Histidinedi chloride—0.1000 gm.—was mixed with 82 cc. of water and 18 cc. of 37 per cent hydrochloric acid in a 300 cc. flask. The solution was then treated with 100 cc. of a 15 per cent aqueous solution of phosphotungstic acid. After standing for 2 hours in the ice chest, crystals had begun to form. They were disintegrated with a glass rod. This gave rise to a rapid and copious precipitation of histidine phosphotungstate. After standing for 24 hours in the ice chest, 1 cc. of the clear supernatant liquid was removed, neutralized, and diluted to 5 cc. Of this solution

0.50 cc. had a color value equivalent to 3.1 mm. (CR–MO)
1.00 " " " " " " " 6.1 " (CR–MO)

This, by table, is equivalent to 0.012 gm. of histidine dichloride per 200 cc. of precipitation liquid. Longer standing in the ice chest produced no change in this value.

The flask was now transferred to an ice bath, where it was kept for 24 hours. At the end of this time, 1 cc. of the clear supernatant liquid was removed, neutralized, and diluted to 5 cc. Of this solution

0.50 cc. had a color value equivalent to 2.1 mm. (CR–MO)
1.00 " " " " " " " 4.2 " (CR–MO)

This, by table, shows that the equivalent of 0.0084 gm. of histidine dichloride—0.00571 gm. of histidine base—was left in solution in the 200 cc. of precipitation liquid.

This experiment shows that, to obtain a most complete and uniform precipitation of histidine phosphotungstate under the

conditions specified by Van Slyke, it is necessary to cool the precipitation mixture in an ice bath. The precipitation, although it is slightly more complete when the concentration of phosphotungstic acid is 7.5 per cent than it is when the concentration is 15 per cent, is not very markedly influenced by the concentration of phosphotungstic acid in the liquid.

The Effect of Other Amino-Acids upon the Colorimetric Determination of Histidine.[11]

Effect of Cystine.

Standard Cystine Solution.—Chemically pure cystine—1.0000 gm.—was dissolved in 30 cc. of N HCl and the resulting solution diluted to exactly 100 cc.

The test solutions were prepared by mixing 1 cc. of a 1 per cent histidine dichloride solution with 2 to 20 cc. of the standard cystine solution and diluting the resulting liquid to 100 cc. The colorimetric determinations were then carried out on 0.20 and 0.40 cc. portions of this test solution.

Table I shows that when cystine and histidine are present in the proportion of 3 (cystine) to 1 (histidine), which is the proportion that is encountered in the cystine-rich keratins, the colorimetric determination of histidine is not interfered with to the slightest extent. The first indication of an interference is obtained when the ratio of cystine to histidine is 6 to 1—a proportion that has not been encountered heretofore in any protein—and under these conditions the interference is only about 3 per cent. A serious interference occurs only when the ratio of cystine to histidine assumes the extremely artificial ratio of 29 (cystine) to 1 (histidine).

From these results we conclude that cystine will not interfere with the colorimetric determination of histidine in any of the known proteins.

[11] We wish to call attention to the fact that the interference percentages given in the following pages are correct only when chemicals of highest purity are used in compounding the alkaline reagent. For the past year we have been using Baker and Adamson's highest purity anhydrous sodium carbonate and sodium nitrite (crystals of 97 to 100 per cent purity) and J. T. Baker's sulfanilic acid.

That the unsaturated sulfur atoms in cystine are responsible for the interference noted for large quantities of this substance is rendered highly probable by the fact that a solution containing equal parts by weight of sodium sulfide and histidine gives rise to a yellowish brown color with *p*-phenyldiazonium sulfonate that is only 40 per cent as intense as that obtained with a pure histidine solution.

TABLE I.

The Effect of Cystine on the Colorimetric Determination of Histidine.

Concentration of histidine per 100 cc.	Concentration of cystine per 100 cc.	Test solution used for the determination.	Theoretical color value.	Color value as determined.	Interference.
mg.	*mg.*	*cc.*	*mm.*	*mm.*	*per cent*
6.8	20	0.20	10.0	10.0	None.
		0.40	20.0	20.0	
6.8	30	0.20	10.0	10.0	None.
		0.40	20.0	20.0	
6.8	40	0.20	10.0	9.7	3
		0.40	20.0	19.5	
6.8	50	0.20	10.0	9.6	4
		0.40	20.0	19.3	
6.8	100	0.20	10.0	9.2	8
		0.40	20.0	18.1	
6.8	200	0.20	10.0	7.6	24
		0.40	20.0	14.7 Color distinct; too yellow.	

Effect of Leucine.

Standard Leucine Solution.—Chemically pure leucine—2.0000 gm.—was dissolved in 20 cc. of N HCl and the resulting solution diluted to exactly 200 cc.

The test solutions were prepared by mixing 1 cc. of a 1 per cent histidine dichloride solution with 10 to 80 cc. of the standard leucine solution and diluting the resulting liquid to 100 cc. The colorimetric determinations were then carried out on 0.20 and 0.40 cc. portions of the test solution.

Table II shows that leucine does not interfere with the colorimetric determination of histidine until the ratio of leucine to histidine has been raised to the extremely exaggerated proportion of 114 (leucine) to 1 (histidine). While one would not, of course, expect the presence of leucine together with the diamino-acids in

TABLE II.

The Effect of Leucine on the Colorimetric Determination of Histidine.

Concentration of histidine per 100 cc.	Concentration of leucine per 100 cc.	Test solution used for the determination.	Theoretical color value (CR-MO).	Color value as determined.	Interference.
mg.	*mg.*	*cc.*	*mm.*	*mm.*	*per cent*
6.8	100	0.20	10.0	10.0	None.
		0.40	20.0	20.0	
6.8	500	0.20	10.0	10.0	None.
		0.40	20.0	20.0	
6.8	800	0.20	10.0	9.6	4
		0.40	20.0	19.4 Color slightly too yellow.	

the phosphotungstic acid precipitate, we believed that it might be desirable to show that the presence of a typical α-amino-acid does not interfere with the quantity and intensity of color produced by histidine.

Effect of Arginine.

Standard Arginine Solution.—A solution containing arginine was prepared from salmon sperm by hydrolyzing with sulfuric acid and precipitating the arginine first with silver nitrate and baryta and then with phosphotungstic acid. The solution of

arginine sulfate obtained by decomposing the phosphotungstate with a cold solution of baryta and removing the barium with a slight excess of sulfuric acid had the following properties.

1. 2 cc. gave 1.70 cc. of amino N_2 at 28° and 752 mm. The solution must, therefore, have contained 0.571 gm. of arginine per 100 cc.

2. Of the above solution, 20 cc. were treated with 0.18 gm. of picrolonic acid dissolved in 3 cc. of alcohol. The picrolonate obtained after drying for 10 hours at 110° weighed 0.2893 gm. and decomposed at 233°.

3. The solution contained no ammonia.

4. When 10 cc. of the solution were mixed with 15 cc. of water and 12.5 gm. of KOH and hydrolyzed for 6 hours as specified by

TABLE III.

The Effect of Arginine on the Colorimetric Determination of Histidine.

Concentration of histidine per 100 cc.	Concentration of arginine per 100 cc.	Test solution used for determination.	Theoretical color value.	Color value as determined.	Interference.
mg.	*mg.*	*cc.*	*mm.*	*mm.*	*per cent*
6.8	115	0.20	10.0	10.0	None.
		0.40	20.0	20.0	
6.8	230	0.20	10.0	9.7	3
		0.40	20.0	19.1	

Van Slyke,[7] sufficient ammonia was evolved to neutralize 6.8 cc. of 0.1 N HCl. The ammonia evolved from this quantity of a 0.571 per cent solution of arginine should have neutralized 6.56 cc. of the 0.1 N acid.

The test solutions were prepared by mixing 1 cc. of a 1 per cent histidine dichloride solution with 20 and 40 cc. of the standard arginine solution and diluting the resulting liquid to 100 cc. The colorimetric determinations were then carried out on 0.20 and 0.40 cc. portions of the test solution.

Table III shows that arginine does not interfere with the colorimetric determination of histidine until the ratio of arginine to histidine has been raised to the proportion of 34 (arginine) to 1 (histidine). Since a ratio of 6 (arginine) to 1 (histidine) is

the largest that has been encountered in any protein heretofore, we conclude that arginine will not interfere with the colorimetric determination of histidine in any of the known proteins.

The Colorimetric Estimation of Histidine in Proteins.

Casein.

Duplicate experiments were carried out on 3 gm. of carefully purified vacuum-dried casein by the method outlined in the introduction. The volume of the test liquid was 500 cc. Of this solution

0.10 cc. had a color value equivalent to 11.7 mm. (CR–MO)
0.20 " " " " " " " 23.4 " (CR–MO)

The color was exactly like that produced by histidine. *The duplicates checked exactly.* This, by table, is equivalent to 0.0000234 gm. of histidine dichloride per 0.10 cc. or 0.117 gm. per entire 500 cc. of test liquid. This is equal to 0.0796 gm. of histidine base in 3 gm. of casein if no correction is made for the solubility of histidine phosphotungstate. We have found that 0.00571 gm. of histidine remains in solution in 200 cc. of precipitation liquid under these conditions; *therefore casein—3 gm.—contains 0.08531 gm. of histidine which is 2.84 per cent.*

Edestin.

Weight dried at 110°, *gm.*	3.0000	
Precipitation volume, *cc.*	200.0	
Volume of the test liquid, *cc.*	1,000.0	
Colorimetric readings:		
0.10 cc. had a color value equivalent to	6.3 mm.	
0.20 " " " " " " "	12.6 "	(CR–MO)
0.30 " " " " " " "	18.9 "	
Histidine dichloride by table, *gm.*	0.126	
" base in test liquid, *gm.*	0.0856	
Solubility correction blank, *gm.*	0.00571	
Histidine in edestin sample, *gm.*	0.09131	
" " " " *per cent.*	3.04	

Horse Hemoglobin.[12]

Sample 1.—Horse hemoglobin—1.9000 gm.—that had been dried *in vacuo* over sulfuric acid for 48 hours, was analyzed for histidine by the method outlined in the introduction. The volume of the pale yellow test liquid was 1,000 cc. Of this solution

0.10 cc. had a color value equivalent to 12.0 mm. (CR–MO)
0.20 " " " " " " " 24.0 " (CR–MO)

The color was exactly like that produced by histidine. This, by table, is equivalent to 0.24 gm. of histidine dichloride—0.1632 gm. of histidine base—for the entire 1,000 cc. of test liquid. This value must be raised by 0.00571 gm.; the solubility correction blank; *therefore horse hemoglobin—1.9000 gm.—contains 0.16891 gm. of histidine which is equal to 8.9 per cent.*

Sample 2.[13]—The air-dried material—2.0000 gm.—was dried *in vacuo* over sulfuric acid for 48 hours. The solid so obtained—1.8664 gm.—was heated to constant weight at 110°. The final product—1.8430 gm.—was then analyzed for histidine by the previously described method. The volume of the test liquid was 1,000 cc. of which

0.10 cc. had a color value equivalent to 11.3 mm. (CR–MO)
0.20 " " " " " " " 22.5 " (CR–MO)

This, by table, is equivalent to 0.226 gm. of histidine dichloride—0.1536 gm. of histidine base—for the entire 1,000 cc. of test liquid. To this must then be added 0.00571 gm., the solubility correction blank; *therefore this sample of horse hemoglobin—1.8430 gm.—contained 0.1593 gm. of histidine which is equal to 8.65 per cent.*

Cat Hemoglobin.

Duplicate analyses were carried out on the same sample.

[12] The four varieties of hemoglobin were kindly furnished by W. H. Welker, Professor of physiological chemistry, University of Illinois, Medical Department.

[13] The two samples of hemoglobin differed both in color and in physical structure.

	First Analysis.	*Second Analysis.*
Weight air-dried, *gm*	2.0000	
" vacuum-dried, *gm*	1.8742	
" dried at 110°, *gm*	1.8561	1.1142
Precipitation volume, *cc*	200.00	100.0
Volume of the test liquid, *cc*	1,000.00	1,000.0
Colorimetric readings:		
0.10 cc. had a color value equivalent to	11.2 mm.	6.8 mm.
0.20 cc. had a color value equivalent to	22.4 "	13.6 "
Histidine dichloride by table, *gm*	0.224	0.136
" base in test liquid, *gm*	0.1522	0.0925
Solubility correction blank, *gm*	0.00571	0.00285
Histidine in hemoglobin sample, *gm*	0.1579	0.09535
Histidine in hemoglobin sample, *per cent*	8.50	8.55

Sheep Hemoglobin.

Weight vacuum-dried, *gm*	2.0000	
Precipitation volume, *cc*	200.0	
Volume of test liquid, *cc*	1,000.0	
Colorimetric readings:		
0.10 cc. had a color value equivalent to	12.5 mm.	(CR–MO)
0.20 " " " " " " "	25.0 "	(CR–MO)
Histidine dichloride by table, *gm*	0.250	
" base in test liquid, *gm*	0.170	
Solubility correction blank, *gm*	0.00571	
Histidine in hemoglobin sample, *gm*	0.17571	
" " " " *per cent*	8.8	

Ox Hemoglobin.[14]

Weight air-dried, *gm*	1.3640	
" vacuum-dried, *gm*	1.2690	
" dried at 110°, *gm*	1.2542	
Precipitation volume, *cc*	150.0	
Volume of test liquid, *cc*	1,000.0	
Colorimetric readings:		
0.10 cc. had a color value equivalent to	7.0 mm.	(CR–MO)
0.20 " " " " " " "	14.0 "	(CR–MO)
Histidine dichloride by table, *gm*	0.140	
" base in test liquid, *gm*	0.0952	
Solubility correction blank, *gm*	0.00428	
Histidine in hemoglobin sample, *gm*	0.09948	
" " " " *per cent*	7.93	

[14] This sample charred slightly while it was being hydrolyzed. The percentage of histidine may, therefore, be somewhat low.

Whole Blood (Normal Human).

Whole blood—exactly 10 cc.—to which a few crystals of ammonium oxalate had been added to prevent coagulation, was mixed with 15 cc. of 37 per cent hydrochloric acid and hydrolyzed in the customary manner. The resulting liquid was then analyzed for histidine by using the method outlined in the introduction.

Precipitation volume, *cc*.....	200.0	
Volume of test liquid, *cc*.....	1,000.0	
Colorimetric readings:		
0.10 cc. had a color value equivalent to..	11.1 mm.	(CR–MO)
0.20 " " " " " " " ..	22.2 "	(CR–MO)
Histidine dichloride by table, *gm*.....	0.222	
" base in test liquid, *gm*.....	0.1516	
Solubility correction blank, *gm*.....	0.00571	
Histidine in 10 cc. of blood, *gm*.....	0.15731	
" " 100 " " " *gm*.....	1.5731	

Blood Serum (Normal Human).

Whole blood—about 30 cc.—was drawn into a centrifuge tube and allowed to clot slowly in the ice chest. The mixture was centrifuged to free the serum as completely as possible from fibrin and blood cells. The perfectly clear, pale yellow serum—exactly 10 cc.—was withdrawn by means of a pipette, mixed with 15 cc. of 37 per cent HCl, and hydrolyzed in the customary manner. The resulting liquid was then analyzed for histidine by using the method outlined in the introduction.

Precipitation volume, *cc*.....	200.0	
Volume of test liquid, *cc*.....	500.0	
Colorimetric readings:		
0.25 cc. had a color value equivalent to...	6.5 mm.	(CR–MO)
0.50 " " " " " " " ...	13.0 "	(CR–MO)
Histidine dichloride by table, *gm*.....	0.0260	
" base in test liquid, *gm*.....	0.01767	
Solubility correction blank, *gm*.....	0.00571	
Histidine in 10 cc. of serum, *gm*.....	0.02338	
" " 100 " " " *gm*.....	0.2338	

STUDIES ON PROTEINOGENOUS AMINES.

VIII. A METHOD FOR THE QUANTITATIVE COLORIMETRIC ESTIMATION OF HISTAMINE IN PROTEIN AND PROTEIN-CONTAINING MATTER.

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In a series of articles published recently[1] we communicated a method by means of which small quantities of histamine, β-imidazolylethylamine, can be accurately determined in a simple

[1] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 497, 521, 539.

synthetic culture medium. We stated at that time, that the method as it was employed in the bacterial metabolism studies was not applicable, in its entirety, to more complex mixtures such as blood and urine. The present communication contains the description of a purely chemical method by means of which the histamine content of tissues and other protein-containing matter can be accurately determined.

I. Description of the Method.

1. Preliminary Treatment of the Material.—A dry solid was usually hydrolyzed immediately without preliminary treatment. A hydrated tissue, like blood, liver, or hypophysis, was mixed with sufficient alcohol to give a final alcohol concentration of 75 per cent. A few drops of acetic acid were then added and the resulting mixture was heated on the water bath for 1 to 2 hours, to extract the free histamine and to coagulate the proteins. The mixture was then cooled and filtered on a Buchner funnel, the residue being washed with 95 per cent alcohol. This divides the material into two fractions, *the alcoholic extract A* and *the alcohol-insoluble residue R*. Each fraction is then freed from alcohol by heating on the water bath after which it is ready to hydrolyze.

2. Hydrolysis.—The dry solid is mixed with ten to twenty parts of 20 per cent hydrochloric acid and hydrolyzed by boiling, under a reflux condenser, for 30 hours over an electrically heated sand bath.

3. Removal of the Hydrochloric Acid.—The hydrochloric acid is removed by distillation *in vacuo* at 60° from the same flask. The residue is finally dried *in vacuo* at 80° for 1 hour.

4. Removal of Ammonia.—The residue is dissolved in ten to twenty parts of water and the solution treated with an excess of lime and a volume of 95 per cent alcohol equal to one-half the volume of the water added. The mixture is then subjected to a distillation *in vacuo* at 40° until its volume has been reduced to about one-half. This removes the ammonia completely.

5. Removal of Humin. The mixture is filtered, on a Buchner funnel, from humin and excess lime, the precipitate being carefully

washed with a large excess of hot water until the washings give a negative Pauly reaction.

6. Preparation of the Phosphotungstates.—The alkaline filtrate is acidified by adding a slight excess of hydrochloric acid. The resulting clear liquid is then evaporated to dryness on the water bath in a glass dish. Because of the variability in the quantity and quality of the original protein-containing matter, a concise statement cannot be made as to the amount of phosphotungstic acid to employ. In general, 4 gm. of phosphotungstic acid are sufficient to precipitate the hexone bases from 1 gm. of dry protein. A quantity of phosphotungstic acid equal to twice the weight of the total solids of a hydrated tissue is usually sufficient to precipitate completely the hexone bases. In all cases the concentration of the hydrochloric acid should be 9 cc. of the 37 per cent acid per 100 cc. of precipitation liquid. The final volume of the precipitation liquid should not exceed 2,000 cc. The phosphotungstates are prepared at water bath temperatures and the mixture is then allowed to cool slowly to room temperature after which it is cooled in an ice bath for 24 hours and filtered with suction. The precipitate is washed with an ice-cold fluid containing 18 cc. of 37 per cent hydrochloric acid and 15 gm. of phosphotungstic acid per total aqueous volume of 200 cc.

7. Decomposition of the Phosphotungstates.—The phosphotungstate *precipitate* which contains the histamine, together with histidine, arginine, lysine, cystine, tyramine, and possibly other amines, is suspended in a large volume of hot water—500 to 4,000 cc.—and treated with an excess of a hot saturated solution of baryta. The resulting mixture is digested for 1 hour on the water bath, after which it is cooled in tap water and filtered on a Buchner funnel, the precipitate being thoroughly washed with hot water. The filtrate is heated on the water bath and freed from excess barium by the careful addition of N H_2SO_4. The mixture is filtered hot through a folded filter. The filtrate, *which should contain a trace of barium*, is then evaporated to dryness in a glass dish. The residue is dissolved in the smallest possible quantity of water, the solution transferred to a graduated cylinder, and made up to the smallest convenient volume—10 to 100 cc. (Liquid 7).

8. Extraction of Histamine with Amyl Alcohol. First Colorimetric Determination.—10 cc. of Liquid 7 are transferred to a glass-stoppered, shake out bottle,[2] mixed with 3 gm. of solid sodium hydroxide, and extracted six times with redistilled[3] amyl alcohol using 20 cc. for each extraction. The combined amyl alcohol extracts are then extracted five times with N H_2SO_4 using 20 cc. for the first and 10 cc. for each of the remaining four extracts. This process is repeated until all Liquid 7 has been extracted.

The combined acid extracts are heated on the water bath and exactly neutralized with baryta. The hot mixture is filtered from barium sulfate, and the filtrate evaporated to dryness in a small glass dish. This residue contains all the histamine. Because of the concentrated character of Liquid 7, a very small quantity of the amino-acids also passes into the amyl alcohol as sodium salts. A colorimetric determination is always positive at this point, because of the presence of histidine. To remove the histidine entirely, the residue obtained above is transferred to the shake out bottle with 10 cc. of water, the solution treated with 3 gm. of solid sodium hydroxide, and extracted with amyl alcohol as above. The sulfuric acid extracts finally obtained are neutralized exactly with baryta. The mixture is filtered from barium sulfate and the filtrate, which should contain no barium, is evaporated to dryness in a small glass dish. The pale yellow residue is dissolved in water and diluted to 25 or 50 cc. Histamine is then estimated colorimetrically in this fraction using the method previously described by us.[1] If the colorimetric test indicates the presence of histamine, the remaining liquid is treated according to Section 9 below. If there is the slightest reason to believe that some of the histidine has again been extracted by the amyl alcohol, which will be apparent from the speed of the color development,[1] a third extraction with amyl alcohol should be carried out. When these extractions are properly conducted the histamine always passes quantitatively into the amyl alcohol.

[2] For a detailed description of this extraction process see Koessler and Hanke,[1] pp. 525–529.

[3] It is best to distill the amyl alcohol *in vacuo*.

9. Precipitation with Silver Nitrate and Baryta. Second Colorimetric Determination.—The liquid obtained under Section 8 is diluted to 100 cc. in a 300 cc. Pyrex flask and mixed with 5 cc. of a 20 per cent silver nitrate solution. To the clear liquid is then added barium hydroxide—8 gm.—dissolved in 50 cc. of warm water. The resulting dark brown mixture is filtered with suction on a platinum cone. The precipitate is washed with 50 cc. of a cold saturated solution of baryta. The filtrate should be *clear*. This divides the material into two fractions, the silver precipitate, which contains the histamine, and the silver filtrate, which although it can contain only traces of histamine seems almost invariably to contain substances that are physiologically active.

The silver precipitate is suspended, with the filter paper, in 50 cc. of water and mixed with 3 cc. of 37 per cent HCl and enough of a 20 per cent Na_2SO_4 solution to remove the barium completely. The mixture is digested on the water bath for 1 hour after which it is filtered and the precipitate washed with hot water. The clear colorless filtrate is neutralized exactly with sodium hydroxide and evaporated to a small volume in a glass dish. The colorless to pale yellow liquid is diluted with water to 25 or 50 cc. Histamine is then estimated colorimetrically in this fraction. If the test shows the presence of histamine, this can be verified biologically on 5 cc. of the liquid and chemically on 20 cc. of the liquid according to Section 10.

This precipitation with silver nitrate and baryta is necessary for two reasons. Test Liquid 8 occasionally contains substances that interfere with the color reaction to such an extent that an accurate determination of histamine is impossible. Such interferring substances appear to be of two kinds; namely, those that *inhibit* the production of color by histamine without giving a color themselves, and those that impart a yellow or green color to the liquid which, of course, makes a perfect match impossible. *These interfering substances were always found to remain in the silver filtrate;* the histamine appears quantitatively in the silver precipitate.

Although one is accustomed to think of histidine, histamine, tyrosine, and tyramine as the only substances that give a positive Pauly reaction, certain of the body tissues appear to contain

small quantities of other substances that give a very similar color reaction. These substances are at least partially removed by the precipitation with silver nitrate and baryta.

10. Extraction of Histamine by Means of Chloroform and Methyl Alcohol. Third Colorimetric Determination.—The liquid obtained under Section 9—20 cc.—is evaporated to dryness *in vacuo* in a 500 cc. long necked, round bottomed flask. The perfectly dry residue is treated with 10 cc. of chemically pure methyl alcohol and 0.50 gm. of potassium hydroxide. The alkaline liquid is then treated with 200 cc. of redistilled chloroform and placed in the ice chest for 15 hours. The mixture is filtered through a small folded filter. The precipitate is washed with 200 cc. of hot chloroform. The chloroform extracts are mixed with a few drops of 37 per cent HCl and subjected to a vacuum distillation to remove the chloroform and the methyl alcohol. The residue is dissolved in water and redistilled *in vacuo* to remove the methyl alcohol completely. The residue finally obtained is dissolved in water, diluted to 20 cc., and examined colorimetrically for histamine.

A final purification of the histamine by means of chloroform in doubtful cases seemed desirable because of the fact that chloroform, although it dissolves histamine, will not dissolve many substances that are soluble in amyl alcohol. We tried first to extract histamine from the dry residue obtained by evaporating a histamine solution—1 cc. of a 1 per cent solution—with 2 gm. of sodium carbonate. The quantity of histamine that passed into the chloroform varied from 60 to 80 per cent of the starting material when the extractions were carried on for 24 hours in a Soxhlet extractor under apparently identical conditions.

When lime was used in place of sodium carbonate, only 27 per cent of the histamine passed into the chloroform.

We tried then to extract histamine from an alkaline aqueous solution by means of chloroform. This proved to be impractical because histamine is too soluble in water and too little soluble in chloroform. Such an extraction would be almost endless.

The following principles are incorporated in the successful process that was described in detail above. Methyl alcohol is a good solvent for histamine, salts of histamine, and potassium

hydroxide. When methyl alcohol is used in conjunction with potassium hydroxide, the *histamine is brought into solution as free base*; so there is no possibility of its being occluded by chloroform-insoluble substances. The addition of chloroform to the methyl alcohol solution of histamine precipitates inorganic salts and most of the potassium hydroxide but the histamine remains in solution.

II. Proof that Histamine can be Quantitatively Recovered by the Methods Described in Sections 6, 7, 9, and 10 of Part I.

1. Histamine Quantitatively Precipitated by Phosphotungstic Acid.—Phosphotungstic acid—37 gm.—and 37 per cent hydrochloric acid—45 cc.—were dissolved in water and the solution was diluted to 500 cc. Histamine dichloride[4] solution—0.50 cc. of a 1 per cent solution—was then added to the above liquid. The resulting mixture was cooled in an ice bath for 2 hours and filtered with suction. The precipitate was suspended in 200 cc. of hot water. The remaining steps were carried out as described in Section 7, Part I. The residue finally obtained was dissolved in water and the solution diluted to 100 cc. Of this solution

0.20 cc. had a color value equivalent to 7.5 mm. (CR–MO)
0.40 " " " " " " 15.0 " (CR–MO)

which, by table, is equivalent to 0.0050 gm. of histamine dichloride, 100 per cent of the amount introduced.

From this experiment it would seem that histamine phosphotungstate is practically insoluble in the precipitation liquid employed at a temperature of 0°. This conclusion was verified by performing a qualitative Pauly reaction on 10 cc. of the filtrate from the histamine phosphotungstate. The reaction was entirely negative.

2. Histamine Quantitatively Precipitated by Silver Nitrate and Baryta.—The liquid obtained above—100 cc.—which still contained 5 mg. of histamine dichloride, was precipitated with silver nitrate and baryta as described in Section 9, Part I. The liquid

[4] See Koessler, K. K., and Hanke, M. T., *J. Am. Chem. Soc.*, 1918, xl, 1716, for a description of the method used in preparing the histamine dichloride.

finally obtained after removing the silver and barium from the silver *precipitate*, was diluted to 100 cc. Of this solution

0.20 cc. had a color value equivalent to 7.5 mm. (CR–MO)
0.40 " " " " " " " 15.0 " (CR–MO)

which, by table, is equivalent to 0.0050 gm. of histamine dichloride for the entire test liquid, 100 per cent of the amount originally introduced.

This experiment proves that histamine is quantitatively precipitated by silver nitrate and baryta under the conditions specified above.

3. Extraction of Histamine by Means of Chloroform and Methyl Alcohol.—Histamine dichloride solution—1.00 cc. of a 1 per cent solution—was evaporated to dryness *in vacuo* in a 500 cc. long necked, round bottomed flask. The dry residue was then treated as described in Section 10, Part I. The residue finally obtained by distillation of the chloroform extracts was dissolved in water and diluted to 100 cc. Of this solution

0.10 cc. had a color value equivalent to 7.2 mm. (CR–MO)
0.20 " " " " " " " 14.4 " (CR–MO)

which, by table, is equivalent to 0.0096 gm. of histamine dichloride, 96 per cent of the amount originally introduced.

III. The Method Applied to Casein with and without the Addition of Histamine.

The search for histamine in a pure protein would seem superfluous if it were not for the fact that Abel and Kubota[5] have recently claimed to have found it, or a physiologically and chemically similar homologue, as a constituent of casein, egg albumin, and edestin. We were not entirely convinced that the physiologically active substance obtained by the above authors was histamine, because they did not identify their substance as histamine chemically and because the presence of this amine as a normal constituent of a pure protein, that has not been allowed to putrefy, seemed rather improbable. Since casein of high purity is easily

[5] Abel, J. J., and Kubota, S., *J. Pharmacol. and Exp. Therap.*, 1919, xiii, 243.

prepared, we decided to use this protein as one check on the accuracy of our method.

Analysis of Casein for Histamine.

Carefully purified casein—40 gm.—prepared from fresh skimmed milk, was mixed with 800 cc. of 20 per cent hydrochloric acid and hydrolyzed by boiling for 30 hours.

The hydrochloric acid, ammonia, and humin were removed as described in Sections 3, 4, and 5, Part I.

The phosphotungstates were precipitated from a total volume of 2,000 cc. with 150 gm. of phosphotungstic acid. The precipitation liquid contained 180 cc. of 37 per cent HCl.

The phosphotungstate precipitate was suspended in 4,000 cc. of hot water and freed from phosphotungstic acid, excess barium, etc., as described in Section 7, Part I. The final volume of Liquid 7 was 20 cc.

The double amyl alcohol extraction of Liquid 7 was carried out as described in Section 8, Part I. The final volume of the test solution was 200 cc., of which 0.20 cc. had no color value and 1.00 cc. gave a pale green color that was quite unlike that of an imidazole and which resembled that produced by ammonia and the aliphatic amines. An accurate comparison with the standard indicator solution was, of course, impossible. By comparing intensities, the green color was found to have an intensity value equivalent to about 3.0 mm. (CR-MO).

There can be little doubt that this sample of casein contained no histamine. If the above *green* color is, nevertheless, ascribed to histamine, 0.0008 gm. of this amine, calculated as hydrochloride, is the maximum amount that could have been present in 40 gm. of casein.

The remainder of the test liquid was evaporated to dryness in a small glass dish. The residue was dissolved in 2 cc. of water and the solution injected into a cat that had been anesthetized and prepared so that a blood pressure and respiratory tracing could be obtained at the same time.[6] The tracing obtained,

[6] In obtaining the blood pressure tracings in this and in the subsequent work we enjoyed the aid of our colleague, Dr. Julian H. Lewis, which we herewith thankfully acknowledge.

Fig. 1, shows that the injection produced a drop in blood pressure entirely similar to that produced by histamine. Some substance or substances are present that behave very similarly to histamine

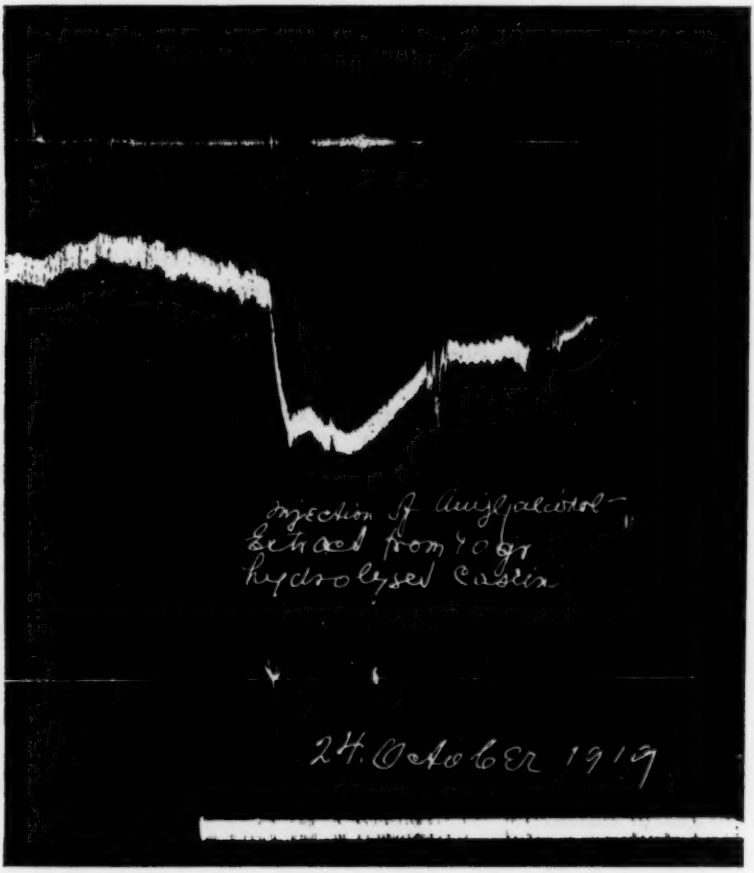

Fig. 1. Blood pressure tracing obtained by injecting the entire amyl alcohol extract and 40 gm. of casein into the femoral vein of a cat.

pharmacologically. We have, therefore, been able to verify the pharmacological findings of Abel and Kubota as illustrated in Fig. 16 of their article; but we cannot conclude with them that casein contains histamine.

Histamine That Has Been Added to Casein Can Be Recovered Quantitatively.

To be certain that our method would recover a small quantity of histamine and that our conclusions concerning casein were correct, another experiment was conducted on 40 gm. of casein exactly like the one that has just been described excepting that 0.01 gm. of histamine dichloride was mixed with the casein before hydrolysis. The final volume of the test liquid was 100 cc., of which

0.05 cc.	had a color value equivalent to	4.0 mm.	(CR–MO)
0.10 "	" " " "	" 8.0 "	(CR–MO)
0.20 "	" " " "	" 15.2 "	(CR–MO)

The color for the 0.05 and 0.10 cc. portions was exactly like that produced by histamine. The color obtained with the 0.20 cc. portion was slightly yellow, and, as can be seen from the above value, there was a slight interference with the color production in this case. Since the values obtained with the two smaller portions checked exactly, the calculations were based upon the 0.10 cc. portion. The reading obtained is equivalent to 0.0107 gm. of histamine dichloride for the entire test liquid which is 107 per cent of the amount actually introduced. Hydrolyzed casein alone gave a green color, the intensity value of which was equivalent to 0.0008 gm. of histamine dichloride. This quantity must, therefore, be subtracted from the above gross value to obtain the amount of histamine dichloride actually recovered which is 0.0099 gm. or 99 per cent of the amount actually introduced.

From these two experiments we conclude that the method gives reliable results and that casein contains no histamine although a substance pharmacologically similar to histamine can be split from casein by acid hydrolysis.

IV. The Method Applied to Human Blood Serum after the Addition of Histamine.

Human blood serum[7]—75 cc.—was mixed with 0.50 cc. of a 1 per cent solution of histamine dichloride. The resulting solution was then treated with 225 cc. of 95 per cent alcohol and a few drops of glacial acetic acid. The mixture was digested for 1 hour on the water bath, cooled to room temperature, and filtered with suction, the coagulum being washed with 95 per cent alcohol. This divided the material into two fractions, *the alcoholic extract A* and *the alcohol-insoluble residue R.*

Alcoholic Extract A.

The alcoholic extract was freed from water and alcohol by distillation *in vacuo* at 50°. The dry residue was treated with 100 cc. of 20 per cent HCl and hydrolyzed as usual. *The material charred considerably* during the process of hydrolysis.

The hydrochloric acid, ammonia, and humin were removed as described in Part I. The phosphotungstates were precipitated from a total volume of 200 cc. with 15 gm. of phosphotungstic acid. The phosphotungstate precipitate was suspended in 800 cc. of hot water and freed from phosphotungstic acid with baryta. The final volume of Liquid 7 was 10 cc. The double amyl alcohol extraction of Liquid 7 was carried out as described in Part I. The final volume of the test solution was 50 cc., of which

0.10 cc. had a color value equivalent to 5.2 mm. (CR–MO)
0.20 " " " " " " " 9.5 " (CR–MO)

The color was exactly like that produced by histamine; but the two readings did not check exactly. A precipitation with silver nitrate and baryta was, therefore, conducted to remove the interfering substances.

The precipitation with $AgNO_3$ and baryta was carried out as described in Section 9, Part I.

The final volume of the test liquid was 50 cc., of which

0.10 cc. had a color value equivalent to 5.2 mm. (CR–MO)
0.20 " " " " " " " 10.3 " (CR–MO)

[7] A sample of this serum was analyzed for histamine by the direct process described on p. 544. The serum contained no histamine.

This, by table, is equivalent to 0.0035 gm. of histamine dichloride.

Alcohol-Insoluble Residue R.

The dried material was mixed with 200 cc. of 20 per cent HCl and hydrolyzed as usual.

The hydrochloric acid, ammonia, and humin were removed as described in Part I.

The phosphotungstates were precipitated from a total volume of 400 cc. with 40 gm. of phosphotungstic acid. The precipitate was suspended in 2,000 cc. of water and freed from phosphotungstic acid and baryta. The final volume of Liquid 7 was 20 cc.

The double amyl alcohol extraction of Liquid 7 was carried out as described in Part I. The final volume of the test solution was 50 cc. of which 1.0 cc. had an intensity value of 4.2 mm. The color was far too yellow for histamine. A further purification of this fraction was effected by means of a silver precipitation.

The precipitation with $AgNO_3$ and baryta was carried out as described in Section 9, Part I. The final volume of the test liquid was 50 cc., of which

0.50 cc. had a color value equivalent to 2.1 mm. (CR–MO)
1.00 " " " " " " 4.2 " (CR–MO)

The color was too yellow for histamine but the time of development was correct for this imidazole. This, by table, is equivalent to 0.0003 gm. of histamine Cl_2 for the entire test liquid.

In this experiment, 3.5 mg. of histamine dichloride appeared in the alcoholic extract and 0.3 mg. appeared in the alcohol-insoluble residue. The total recovery was 3.8 mg. Since 5 mg. were originally introduced, 1.2 mg. of histamine dichloride were lost somewhere in the process. We have previously shown[1] that histamine is readily adsorbed by charcoal. The alcoholic extract A charred considerably during the process of hydrolysis; so it is possible that the 1.2 mg. of histamine Cl_2 were adsorbed by the charcoal formed at this time. To prove that this statement is correct, the following experiment was carried out in which the possibility of charcoal formation was entirely eliminated.

The same sample of blood serum—75 cc.—was mixed with 0.50 cc. of a 1 per cent solution of histamine dichloride. The resulting solution was treated with 225 cc. of 95 per cent alcohol and a few drops of glacial acetic acid. The mixture was digested

for 1 hour on the water bath, cooled to room temperature, and filtered with suction, the coagulum being washed with 95 per cent alcohol. The alcoholic extract A was evaporated to dryness in a small glass dish. The residue was transferred to the shake out bottle with 10 cc. of water and the solution treated with 3 gm. of solid NaOH.

A double amyl alcohol extraction was then carried out[8] as described in Section 8, Part I. The final volume of the test liquid was 100 cc., of which

0.20 cc. had a color value equivalent to 7.2 mm. (CR–MO)
0.40 " " " " " " 14.4 " (CR–MO)

The color was exactly like that produced by histamine. This, by table, is equivalent to 0.0048 gm. of histamine dichloride. Since the alcohol insoluble residue was previously proved to adsorb the equivalent of 0.0003 gm. of histamine dichloride, the total recovery in this case was 0.0051 gm. as against 0.0050 gm. actually introduced.

This leaves little doubt that the 1.2 mg. of histamine dichloride that were lost in the hydrolysis experiment had been adsorbed by the charcoal that was formed during the process of hydrolysis.

SUMMARY.

1. A colorimetric method is described by means of which quantities of histamine (β-imidazolylethylamine) as small as 0.1 mg. can be accurately determined in protein and protein-containing matter.

2. The presence of histamine could not be demonstrated in 40 gm. of casein by this method.

3. Casein contains a depressor substance that is similar to histamine pharmacologically.

4. Histamine, that has been added to casein before the latter is hydrolyzed, can be recovered quantitatively.

5. The presence of histamine could not be demonstrated in 75 cc. of human blood serum by this method.

6. Histamine that has been added to the blood serum before the latter is hydrolyzed can be recovered quantitatively.

[8] We wish to call attention to the fact that in this case Steps 2 to 8 of the general process described in Part I have been eliminated. Although this process is very simple and rapid, it can be used only to estimate loosely combined histamine. Peptamine histamine would probably not pass into the amyl alcohol.

STUDIES ON PROTEINOGENOUS AMINES.

IX. IS HISTAMINE A NORMAL CONSTITUENT OF THE HYPOPHYSIS CEREBRI?

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INTRODUCTION.

The physiological activity of extracts of the hypophysis has been a subject of constant investigation since Oliver and Schäfer discovered in 1895 that such extracts had the power of raising the blood pressure of animals on intravenous injection. Howell, 3 years later, brought experimental proof that it was only the posterior part of the hypophysis which possessed this pressor action. This elevation of pressure seemed to result from the direct action of the active substance upon the involuntary muscle of the heart and arteries without the intervention of the sympathetic nervous system. This specific affinity of the active principle of the hypophysis for the smooth muscle fiber cell was confirmed anew through the important discovery of Dale that pituitary extracts have the faculty of producing intense contractions of the uterus. The detection of this oxytocic action lead to the introduction of pituitary extracts into human therapeutics. Since then, the isolation of the active principle of the hypophysis has been repeatedly attempted by biochemists.[1] Although our knowledge has been greatly increased by these investigations, the actual chemical composition of the active principle of the hypophysis is still unknown.

[1] For a review of the work and literature on the chemistry of the pituitary gland see Crawford, A. C., *J. Pharmacol. and Exp. Therap.*, 1920, xv, 81.

There are two facts that seem to stand out from the sum total of these investigations. The active principle of the gland behaves like an amine, and it seems always to be associated with an imidazole derivative. These two chemical properties claimed for the active principle of the hypophysis, in conjunction with the physiological activity of the extracts on the uterus, suggested a connection with β-imidazolylethylamine (histamine). Since this amine is derived from histidine by decarboxylation, Barger suggested that "The pituitary active principle is possibly a polypeptid like derivative of histidine." Decarboxylated polypeptides of this type have been synthetized by Guggenheim who proposed for them the name peptamines. They show the same physiological behavior as the amine from which they are derived; but to a much less degree.

This whole question seemed to be definitely settled in a very simple manner when Abel and Kubota[2] published a paper in which they state that "histamine is the plain muscle-stimulating and depressor constituent of the posterior lobe of the pituitary gland." This statement is based upon the actual isolation and identification of 18 mg. of histamine dipicrate from 1 pound of *dried* substance of the whole pituitary gland. It is difficult to accept the conclusions of these authors, because of the differences in chemical as well as physiological behavior of pituitary extracts and histamine.

There is a widespread conception that histamine is a very labile substance. Thus, for example, Myers and Voegtlin[3] suggested recently in connection with some work on the chemical isolation of vitamines that the physiological activity of a crystalline product containing histamine or histamine-like substances was destroyed by drying. This certainly does not hold true for histamine; for this amine loses none of its physiological activity when it is dried at room temperature or at a temperature of 100° in air or *in vacuo*. We have shown in a previous publication[4] that histamine, when it is heated on the boiling water bath with concentrated sodium hydroxide for 7 hours, is only destroyed to

[2] Abel, J. J., and Kubota, S., *J. Pharmacol. and Exp. Therap.*, 1919, xiii, 243.

[3] Myers, C. N., and Voegtlin, C., *Proc. Nat. Acad. Sc.*, 1920, vi, 3.

[4] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 539.

the extent of 7.5 per cent, while 10 hours of boiling with hot concentrated hydrochloric acid leaves the histamine unchanged. Guggenheim[5] showed in 1914 that the pressor and oxytocic properties of "pituglandol" are completely destroyed even by dilute alkali—2.0 N NaOH—at room temperature, while the physiological activity of histamine on bronchi and blood pressure was not in the least impaired by the same treatment. The differences in physiological activity of the extracts of the hypophysis and of histamine, with especial regard to their broncho-constrictor action, have been admirably discussed by Jackson and Mills.[6]

Experiments on cases of diabetes insipidus in man have shown that the reaction of the pituitary extract in suppressing the polyuria for days cannot be duplicated by injections of histamine (Rowntree).

The typical urticaria-like wheal produced on application of histamine to the scarified skin of man cannot be obtained with pituitary extracts.[7]

We have developed quantitative methods, in our laboratory, for the estimation of histamine and other imidazoles in tissues and were applying these methods to a detailed study of the presence of histamine in the animal organism at the time of the appearance of the article by Abel and Kubota. Considering the far reaching importance of the hypophysis problem it seemed logical to extend our research to the hypophysis. From the results of this part of our work we have to conclude that *the perfectly fresh hypophysis contains no histamine*. Still there can be no question that Abel and Kubota isolated histamine from the pound of pituitary substance which they secured in a dry state on the market. The question is, how did the histamine get into the pituitary material used by these investigators? It is a well known fact[4] that histamine is readily formed from histidine by the action of certain putrefactive microorganisms; and histidine is a normal constituent of practically all protein of animal origin. The method used at the slaughter houses for

[5] Guggenheim, M., *Biochem. Z.*, 1914, lxv, 189.

[6] Jackson, D. E., and Mills, C. A., *J. Lab. and Clin. Med.*, 1919, v, 1.

[7] Sollmann, T., and Pilcher, J. D., *J. Pharmacol. and Exp. Therap.*, 1916–17, ix, 309.

preparing dried glandular products gives ample opportunity for changes, both autolytic and bacterial. Our work on the relation of histamine to peptone shock (page 567) emphasizes again how essential it is to control bacterial action and how imperative it is to work only with material that has been prepared by the investigators themselves.

We have been able to demonstrate the presence of histamine in the liver and feces of one dog by the method used in the present investigation and we expect to report on this phase of the work in the near future; but we were unable to demonstrate the presence of the slightest trace of histamine in the hypophysis. *On the basis of this work, the claim of Abel and Kubota to have found in histamine the plain muscle stimulating and depressor constituent of the posterior lobe of the pituitary gland seems to us untenable.*

EXPERIMENTAL.

Collection of the Material.

The perfectly fresh glands—beef—were collected at Swift and Company's Chicago plant. They were trimmed free from extraneous tissue, weighed, and dropped into boiling absolute alcohol approximately 25 minutes after the animal had been struck. The total weight of the moist glands was 346 gm.

The alcohol was removed by distillation *in vacuo* at 40°. The residue was put through a food chopper. The finely divided material was then digested for 2 hours with 2,000 cc. of 75 per cent alcohol to which a few cc. of glacial acetic acid had been added. The mixture was cooled and filtered with suction, the residue being washed thoroughly with 95 per cent alcohol. This divided the material into two fractions, the alcoholic extract A and the alcohol-insoluble residue R.

The Alcoholic Extract A.

The alcohol and water were removed by distillation *in vacuo* at 50°. The residue was emulsified with hot water and diluted to 140 cc. in a graduated cylinder. This fraction might contain histamine either free or in the form of a peptamine. Since it seemed desirable to know not only how much histamine was

present but also the form in which it was present in the gland, this fraction was divided into two equal parts. One-half was extracted directly with amyl alcohol without hydrolysis, the other half was hydrolyzed, treated with phosphotungstic acid, and then extracted with amyl alcohol.

Loosely Bound Histamine. Non-Hydrolyzed Alcoholic Extract A.—This fraction which was not hydrolyzed, was extracted with amyl alcohol, 10 cc. at a time, as described in Part I of the preceding article, page 544. The combined amyl alcohol extracts were then extracted with N H_2SO_4,[8] the acid extracts neutralized with baryta, the mixture was filtered from $BaSO_4$, and the filtrate evaporated to dryness in a glass dish. The residue was transferred to a shake out bottle with 10 cc. of water. Sodium hydroxide—3 gm.—was added and the liquid extracted six times with 20 cc. of amyl alcohol. The combined amyl alcohol extracts were extracted with N H_2SO_4, using 20 cc. for the first and 10 cc. for each of the remaining four extracts. The acid extracts were neutralized with baryta, the mixture was filtered from $BaSO_4$, and the filtrate, which was free from barium, was evaporated to dryness in a small glass dish. The residue was dissolved in water, the solution transferred to a 50 cc. graduated precision cylinder, and diluted with water to 50 cc. Of this solution

0.20 cc. had a color value equivalent to 3.1 mm. (CR-MO)
0.50 " " " " " " " 7.5 " (CR-MO)

The color resembled that produced by histamine excepting in its time of development which was too rapid for histamine. This, by table, is equivalent to 0.001 gm. of histamine dichloride for the entire test liquid.

The remaining liquid was subjected to a phosphotungstic acid precipitation as described in Sections 6, 7, and 8, Part I, of the preceeding paper. A colorimetric determination showed that *all* the color-producing substance had been precipitated by the phosphotungstic acid.

[8] The details of this process are given in Section 8, Part I, of the preceding article.

The liquid was then subjected to a precipitation with silver nitrate and baryta as described in Section 9, Part I, of the preceding paper.

The fraction *precipitated by silver* was finally brought to a volume of 50 cc. Of this solution

0.50 cc. had no perceptible color value.
1.00 cc. gave a very faint green color.

This would indicate that the color-producing substance that was soluble in amyl alcohol and precipitated by phosphotungstic acid *could not have been histamine* because *it did not give an insoluble silver compound.*

The silver filtrate, which contained the color-producing substance, did not contract the excised virgin guinea pig uterus.

We conclude, therefore, that the perfectly fresh hypophysis contains no loosely bound or free histamine.

Peptamine Histamine. Hydrolyzed Alcoholic Extract A.—This fraction—70 cc., one-half of the entire alcoholic extract A—was mixed with 70 cc. of 37 per cent HCl and hydrolyzed by boiling for 30 hours over an electrically heated sand bath. The material was freed from HCl, NH_3, and humin, precipitated with phosphotungstic acid,[9] and extracted with amyl alcohol as described in Sections 1 to 9, Part I, of the preceding paper.

The final volume of test Liquid 8 was 100 cc., of which

0.50 cc. had a color value equivalent to 2.7 mm. (CR–MO)
1.00 " " " " " " 5.5 " (CR–MO)

The color resembled that produced by histamine excepting in its time of development which was too rapid for histamine. This, by table, is equivalent to 0.00074 gm. of histamine dichloride for the entire test liquid.

The remainder of the above solution was subjected to a precipitation with silver nitrate and baryta as described in Section 9, Part I, of the preceding paper. The fraction *precipitated by silver* was finally brought to a volume of 50 cc., of which

0.50 cc. had a color value equivalent to 2.0 mm. (CR–MO)
1.00 " " " " " " 3.8 " (CR–MO)

[9] The volume of the precipitation liquid was 250 cc. 20 gm. of phosphotungstic acid were employed.

This, by table, is equivalent to 0.00025 gm. of histamine dichloride for the entire test liquid.

The remaining liquid was evaporated to dryness. The residue was dissolved in 5 cc. of water. *Of this solution, 1 cc. did not contract the excised virgin guinea pig uterus.* This proves that *the alcoholic extract from the hypophysis contained no histamine.* The slight color value of this fraction, which we found to be equivalent to 0.00025 gm. of histamine dichloride, must therefore have been due to some physiologically inert substance. *It could not have been due to histamine.*

Recapitulation.

The 75 per cent alcoholic extract from 346 gm. of fresh hypophysis does not contain histamine.

The Alcohol-Insoluble Residue R.

The dry residue, which weighed 132 gm., was mixed with 1,500 cc. of 20 per cent hydrochloric acid and hydrolyzed in the customary manner.

The material was freed from HCl, NH_3, and humin, and precipitated with phosphotungstic acid[10] as described in Sections 1 to 8, Part I, of the preceding paper. The residue finally obtained from the phosphotungstate *precipitate*, was nearly solid and large in volume. Fully 300 cc. of water were required to bring this residue into solution. This would have required so many amyl alcohol extractions that a new procedure was adopted. The solution was treated with 20 gm. of anhydrous sodium carbonate. The resulting liquid was evaporated on the water bath and the residue dried for 48 hours *in vacuo* over sulfuric acid. The perfectly dry, brown solid was then extracted with chloroform for 72 hours in a Soxhlet extractor. This divided the material into two fractions, the *chloroform extract*, which should contain *most* of the histamine, and the *chloroform-insoluble residue*.

[10] The volume of the precipitation liquid was 2,000 cc. 250 gm. of phosphotungstic acid were employed.

The Chloroform Extract.

The combined chloroform extracts were evaporated on the water bath. The residue was dissolved in water and diluted to 50 cc. Of this solution 1.00 cc. gave a faint *green* color that could most certainly not have been produced by histamine. The solution was evaporated to dryness. The residue was dissolved in water and diluted to 5 cc. *Of this solution, 1.00 cc. did not contract the excised virgin guinea pig uterus.*

This proves that the chloroform extract, which should certainly have contained *some* histamine if any had been present in the original solid, was entirely free from histamine.

The Chloroform-Insoluble Residue.

To be perfectly certain that the histamine had not been adsorbed by the solids in this residue so that none of it was extracted by the chloroform, the solid was extracted three times with hot 95 per cent alcohol, using 200 cc. for each extraction. The combined alcoholic extracts were evaporated on the water bath. The residue was dissolved in water, the solution treated with sodium hydroxide, and the alkaline liquid extracted as usual with amyl alcohol. The test liquid finally obtained had a volume of 50 cc. of which

0.20 cc. had an intensity value equivalent to 5.0 mm. (CR–MO)
1.00 " " " " " " " 8.0 " (CR–MO)

As can be readily seen, the above values are worthless because the 1 cc. portion gave almost the same color as the 0.20 cc. portion. To eliminate the interfering substances, a silver precipitation was now conducted as described in Section 9, Part I, of the preceding paper. The final volume of the test liquid was 25 cc., of which

0.10 cc. had a color value equivalent to 8.2 mm. (CR–MO)
0.20 " " " " " " " 16.2 " (CR–MO)

The color was brown, attained its full intensity almost immediately, and was perfectly stable so that it did not resemble the color produced by histamine in any particular. If, nevertheless,

the color value is calculated as histamine dichloride, the presence of 0.00275 gm. of this substance is indicated.

That the above color value was not due to histamine was proved as follows:

1. *1 cc. of the test liquid did not contract the excised virgin guinea pig uterus.*

2. That the substance responsible for the color production was different from histamine chemically was proved by subjecting the remainder of the liquid—23 cc.—to the chloroform methyl alcohol purification described in Section 10, Part I, of the preceding paper. The residue finally obtained was dissolved in water and diluted to 23 cc. Of this solution

0.50 cc. had a color value equivalent to 8.6 mm. (CR–MO)
1.00 " " " " " " 17.0 " (CR–MO)

The color was like that previously obtained and not like that produced by histamine. By table, this would be equivalent to 0.00057 gm. of histamine dichloride for the entire test liquid, which is the maximum amount of this amine that could have been present in the entire 346 gm. of moist glands originally employed. That histamine could not have been responsible for even this slight color value was previously proved by the fact that the liquid after the silver precipitation did not contract the excised virgin guinea pig uterus.

CONCLUSION.

Perfectly fresh beef hypophysis does not contain histamine.

STUDIES ON PROTEINOGENOUS AMINES.

X. THE RELATION OF HISTAMINE TO PEPTONE SHOCK.

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INTRODUCTION.

The discovery that the intravenous injection of proteose-peptone into animals produces profound systemic disturbances of shock-like character was made by Schmidt-Muhlheim[1] and Fano[2] in the laboratory of Ludwig. Ludwig and his pupils were studying the mode of absorption of proteins from the intestinal tract, their fate, and their resynthesis in the animal organism. In an endeavor to settle the question whether proteins are mainly absorbed as proteoses or peptones or as amino-acids, proteose-peptone solutions were injected into the circulation of animals (dogs). While the primary inquiry was not definitely answered by these experiments, they resulted in the observation that the animals injected showed a very marked fall of blood pressure, acceleration of lymph flow, incoagulability of the blood, subnormal temperature, increased frequency of respiration, and a state of loss of consciousness (peptone shock).

In interpreting this toxic action of the proteose-peptones two distinct views have crystallized from the work of a large number of investigators.[3] One group believes that the toxicity of the proteose-peptone is a property inherent in the molecular struc-

[1] Schmidt-Muhlheim, A., *Arch. Physiol.*, 1880, 33.

[2] Fano, *Arch. Physiol.*, 1881, 277.

[3] For the complete literature of the subject see Chittenden, R. H., Mendel, L. B., and Henderson, Y., *Am. J. Physiol.*, 1898–99, ii, 142; and especially Underhill, F. P., and Hendrix, B. M., *J. Biol. Chem.*, 1915, xxii, 443.

ture of these products of protein digestion. The other group ascribes their toxic action to the presence of contaminating substances which can be extracted from the proteose-peptone mixture; the latter thus freed from the supposedly true poison is said to have no physiological activity. Brieger's peptotoxin,[4] Pick and Spiro's peptozyme,[5] and Popielski's vasodilatin[6] are substances of undetermined chemical composition that are supposed to represent the poison which can be separated from the peptone. Though the work of Nolf, Underhill, Zunz, and Gibson seemed to have proved definitely that proteose-peptones of vegetable as well as of animal origin are toxic in themselves, the doctrine of a separable toxin in peptone has been recently revived by Abel and Kubota,[7] who state that *histamine is the toxic agent of Witte's peptone.* A possible relation of β-imidazolylethylamine to peptone shock had been previously suggested by Dale and Laidlaw,[8] who emphasized the great similarity in the symptoms of peptone shock to those produced by the injection of histamine. The whole symptom-complex of peptone intoxication was revived and the older work on this subject brought again to the attention of the biologists through the studies of Biedl and Kraus[9] on anaphylaxis. They recalled first the striking resemblance of the symptoms produced in animals sensitized to a certain protein on reinjection of this protein with the syndrome obtained by Ludwig's pupils on peptone injection. In a later publication they consider anaphylaxis as a true peptone intoxication. Dale and Laidlaw never regarded the correspondence of symptoms as a sufficient basis for theoretical speculations. That they are still of the same opinion can be seen from the following statement in their paper on histamine shock.[10]

[4] Brieger, L., *Z. physiol. Chem.*, 1882–83, vii, 274. For a criticism of Brieger's peptotoxin work, see Salkowski, E., *Virchows Arch. path. Anat.*, 1891, cxxiv, 409.

[5] Pick, E. P., and Spiro, K., *Z. physiol. Chem.*, 1900–01, xxxi, 235.

[6] Popielski, L., *Arch. ges. Physiol.*, 1909, cxxvi, 483.

[7] Abel, J. J., and Kubota, S., *J. Pharmacol. and Exp. Therap.*, 1919, xiii, 243.

[8] Dale, H. H., and Laidlaw, P. P., *J. Physiol.*, 1910–11, xli, 318.

[9] Biedl, A., and Kraus, R., *Wien. klin. Woch.*, 1909, xxii, 363. Kraus, R., and Levaditi, C., Handbuch der Technick und Methodik der Immunitatsforschung, Jena, 1911, i, 255–290.

[10] Dale, H. H., and Laidlaw, P. P., *J. Physiol.*, 1919, lii, 355.

"The existence of these points of community, in the action of substances so utterly unrelated chemically as histamine and certain metallic ions, forbids any assumption that the production of similar effects, by unknown constituents of some organ or tissue, indicates the presence therein of histamine itself, or of any substance chemically related to it. The similarity depends on the fact that all act on the endothelium, and produce in it changes probably of the same general type. A hint, as to what the nature of these changes may be, is possibly provided by the fact that the anaphylactic reaction, more especially in those species in which histamine exhibits this type of action, also presents the picture of an acute endothelial poisoning. All available evidence goes to show that the anaphylactic antibody is of the nature of a "precipitin," the interaction of which with the corresponding antigen results in a change in the state of dispersion of the colloidal particles."

Abel and Kubota, though well acquainted with Dale and Laidlaw's paper, take the view that histamine is present wherever living protoplasm exists, or at least wherever protoplasm is killed. Believing that it makes its appearance wherever a true protein is disrupted by enzymes, acids, or other hydrolytic agents, they believe they have demonstrated its presence in ereptone, Witte's peptone, casein, and edestin. Traumatic shock, anaphylaxis, Vaughan's protein poison, the active principle of the posterior part of the hypophysis, and many other questions of fundamental importance to medicine they believe are settled on the basis of the presence and physiological activity of histamine. To bring about the solution of all these questions in such a simple manner is so suggestive and attractive that it is only with a certain sense of reluctance that we venture the opinion that the far reaching conclusions of Abel and Kubota are not justified by the data of their experimental work. In only one case have they actually demonstrated the presence of histamine; namely, in a dried commercial sample of hypophysis glands.

We have discussed the relation of histamine to the hypophysis problem in the preceding paper. In working on the presence of histamine in protein products Abel and Kubota used two commercial preparations, ereptone and Witte's peptone. Physiological tests made with solutions prepared from an impure picrate led them to believe that the shock poison of ereptone and Witte's peptone is histamine. The results obtained with these commercial preparations are directly transferred to peptone

shock in general. Bacterial putrefaction was not excluded in the preparation of the protein derivatives used; thus the fundamental question, so far reaching for this whole problem and recognized as such by the above authors in the introduction to their paper,[11] namely if histamine can be formed in the absence of bacteria by the enzymatic activity of organ cells alone, is still unanswered.

Vaughan's poisonous protein fraction is not identical with histamine, for its toxicity is completely destroyed by boiling with dilute (3 per cent) hydrochloric acid,[12] while heating histamine with concentrated hydrochloric acid for 10 hours on the boiling water bath leaves the imidazole compound intact,[13] and does not impair its toxicity.

To expect a similarity in physiological action from compounds having a similar chemical structure is logical and justified by evidence; but to conclude from a similar pharmacodynamic action to the identity of chemical structure or the invariable presence of one and the same substance is a perilous undertaking.

Plan of Procedure.

The main postulates of an endeavor to clear up the relation of histamine to peptone shock seemed to be:

1. The preparation of a proteose-peptone under sterile conditions. Since certain microorganisms have the faculty of forming histamine from protein matter, no conclusions can be drawn regarding the origin of histamine found in a sample of peptone if this postulate is not fulfilled. We controlled the absence of bacteria during the time of the preparation of our peptone by daily preparations of aerobic and anaerobic cultures.

2. The peptone must be physiologically active. That this was actually the case can be seen by consulting Figs. 1 and 2.

[11] Abel and Kubota,[7] pp. 247–249.

[12] Underhill, F. P., and Hendrix, B. M., *J. Biol. Chem.*, 1915, xxii, 465.

[13] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 521.

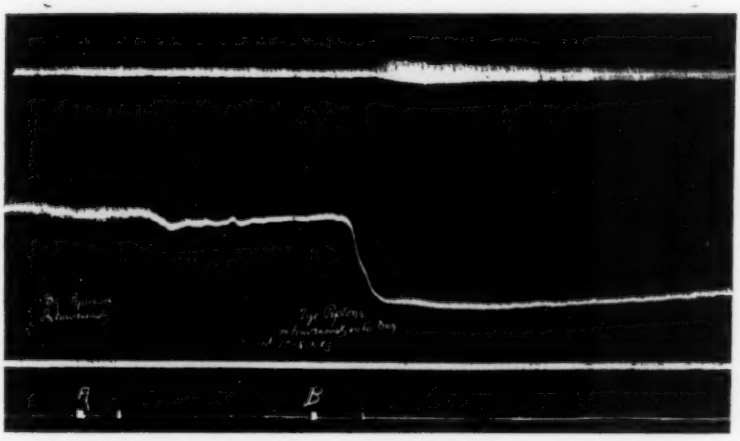

FIG. 1. (A) Injection of pepsin. (B) Injection of 2 gm. of histamine-free peptone prepared from fibrin.

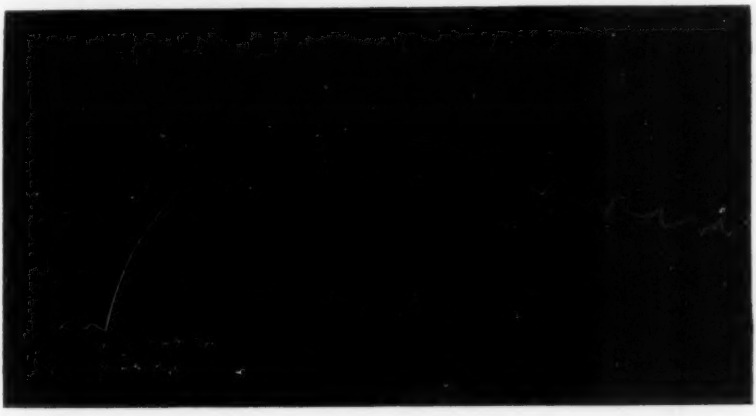

FIG. 2. Contraction of the excised virgin guinea pig uterus produced by 0.1 gm. of a histamine-free, fibrin peptone. The uterus was suspended in 100 cc. of Locke-Ringer solution.

EXPERIMENTAL.

Preparation of Histamine-Free Peptone from Fibrin.[14]

Well washed blood fibrin—275 gm.—was treated with 11,000 cc. of 0.2 per cent sodium hydroxide. The mixture was covered with a layer of toluene and allowed to stand at room temperature for 8 days. The opalescent mixture was filtered through several layers of toweling. The filtrate—10,000 cc.— was diluted with 10,000 cc. of water. Acetic acid—0.5 per cent—was then added in small portions until a perfect flocculation had occurred. The precipitate was washed four times, by decantation, with distilled water and was then largely freed from water by filtration on a Buchner funnel. The residue obtained—116 gm.—was mixed with 350 cc. of 0.4 per cent hydrochloric acid and 30 cc. of a 0.1 per cent pepsin solution in 0.4 per cent hydrochloric acid. The mixture was covered with toluene and incubated at 37° for 1 week. That the liquid was free from living microorganisms throughout this entire period was proved by repeated aerobic and anaerobic cultures. The resulting mixture was nearly neutralized with a 10 per cent sodium carbonate solution, heated to boiling, and filtered through a large, water-soaked, folded filter paper. The filtrate was evaporated to dryness *in vacuo* over sulfuric acid, at room temperature. The dry, pale yellow powder weighed 14.5 gm. This will be referred to as the "fibrin peptone."

Analysis of Fibrin Peptone for Histamine.

5 gm. of the peptone were dissolved in 100 cc. of 20 per cent hydrochloric acid and hydrolyzed by boiling for 30 hours over an electrically heated sand bath. The acid, ammonia, and humin were removed as previously described.[15] The phosphotungstates were precipitated from a total volume of 400 cc. with 40 gm. of

[14] We are indebted to Dr. F. C. Koch, Department of Physiological Chemistry, University of Chicago, for the details of the method used in the preparation of the peptone.

[15] A detailed description of the method used in the estimation of histamine in protein-containing matter has been reported (Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1920, xliii, 543).

phosphotungstic acid. The phosphotungstate precipitate was freed from phosphotungstic acid, etc. The residue finally obtained was subjected to a double amyl alcohol extraction. The volume of the test liquid was 50 cc. of which, tested by our meth-

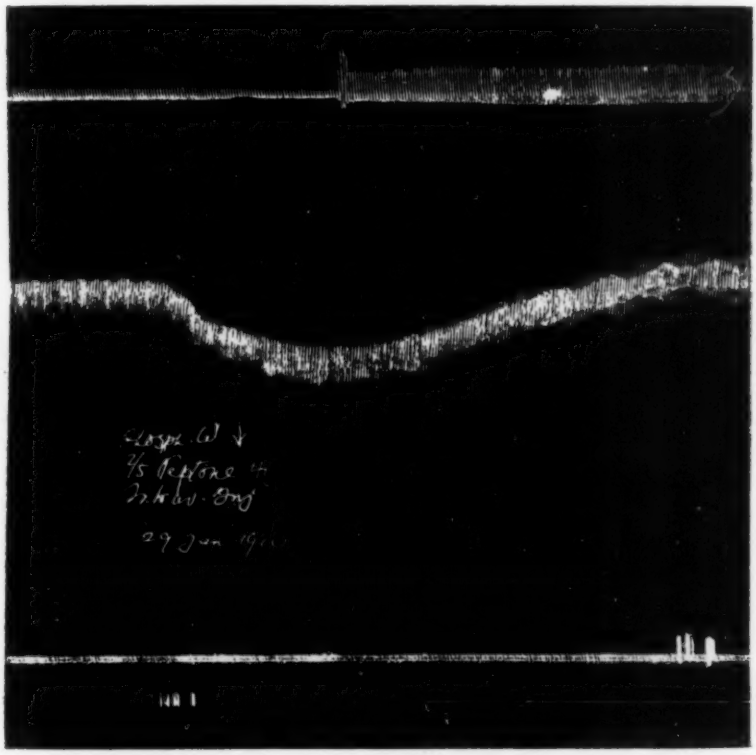

FIG. 3. The peptone was hydrolyzed, precipitated with phosphotungstic acid, and the phosphotungstate precipitate extracted twice with amyl alcohol from an alkaline solution. The liquid was free from histamine. The 2 cc. injected represent the amyl alcohol extract from 2 gm. of peptone.

ods, 0.50 cc. gave no perceptible color and 1.00 cc. gave a very faint green color. *This proves that the above peptone was entirely free from histamine.*

The remaining liquid was evaporated on the water bath. The dry residue was dissolved in 5 cc. of water. Of the resulting solution, *1.00 cc. did not contract the excised virgin guinea pig uterus*; which substantiates the conclusion previously arrived at that histamine was absent. Of the same solution, 2 cc. were injected into the femoral vein of a dog. Fig. 3 shows that this injection produced a slight drop in blood pressure which must, therefore, have been produced by some substance not identical with histamine.

A Typical Peptone Shock from a Histamine-Free Peptone.—2 gm. of the above peptone, that had been proved to contain no histamine, were dissolved in 6 cc. of isotonic salt solution and injected into the femoral vein of a dog that had been anesthetized and arranged so that blood pressure and respiratory tracings could be obtained. As can be seen by examining Fig. 1, this injection gave rise to a typical peptone shock. A morphological blood examination showed before the injection of the peptone, hemoglobin 88, leucocytes 20,600, erythrocytes 6,250,000; 1 hour after the injection, hemoglobin 108, leucocytes 5,700, and erythrocytes 7,200,000.

The Action of Peptone on the Unstriated Muscle of the Guinea Pig Uterus.—That Witte's peptone has the property of contracting the isolated uterus of the virgin guinea pig in a manner that is indistinguishable from that of histamine has been shown by Dale and Laidlaw.[8] Abel and Kubota, who worked with a chloroform extract from Witte's peptone, ascribe this oxytocic reaction of peptone to histamine. That this action is also inherent in the structure of the peptone molecule and not dependent upon the presence of histamine, we showed by using our fibrin peptone, which had been proved to be free from histamine.

5 cc. of a 2 per cent solution of fibrin peptone—0.1 gm. of peptone—were introduced into 100 cc. of Locke-Ringer solution in which an excised guinea pig uterus was suspended. A typical contraction of the uterus was obtained which is illustrated by the curve of Fig. 2.

To prove that the reactions were produced by the peptone obtained from fibrin and not by some constituent of the pepsin employed in the preparation of the peptone, 30 cc. of a 0.1 per cent pepsin solution in 0.4 per cent HCl were incubated for

1 week, neutralized with sodium carbonate, evaporated to dryness, dissolved in 2 cc. of water, and injected into the femoral vein of a dog. An examination of Fig. 1, Part A, shows that this injection did not produce a fall in blood pressure.

Analysis of Witte's Peptone for Histamine.—Witte's peptone—5 gm.—was hydrolyzed, freed from hydrochloric acid, ammonia, and humin, precipitated with phosphotungstic acid, and extracted with amyl alcohol, the details being identical with those just described for the fibrin peptone. The volume of test Liquid 8 was 25 cc., of which

0.50 cc. had an intensity value equivalent to 2.6 mm. (CR–MO)
1.00 " " " " " " " 4.1 " (CR–MO)

The color was orange-yellow and hence difficult to match against the standard indicator solution. The remaining liquid—23.5 cc.—was subjected to a precipitation with silver nitrate and baryta.[15] The volume of test Liquid 9 was 23.5 cc., of which

0.50 cc. had a color value equivalent to 2.5 mm. (CR–MO)
1.00 " " " " " " " 5.0 " (CR–MO)

The color was somewhat more yellow than that produced by histamine; but the time of development was correct for this imidazole. This, by table, is equivalent to 0.0001675 gm. of histamine dichloride for the entire test liquid.

The remaining liquid—22 cc.—was evaporated on the water bath. The residue was dissolved in 5 cc. of isotonic salt solution. Of this solution, *1.00 cc. contracted the excised virgin guinea pig uterus.* The curve obtained, Fig. 4, was exactly like that obtained with a solution of pure histamine dichloride.

This sample of Witte's peptone contained the equivalent of 0.00335 gm. of histamine dichloride per 100 gm. of peptone.

Histamine that Has Been Added to Peptone Can Be Recovered Quantitatively.—This experiment was a duplicate of the one that has just been described excepting that 0.0050 gm. of histamine dichloride was added to the 5 gm. of Witte's peptone before hydrolysis. The final volume of test Liquid 8 was 100 cc., of which

0.20 cc. had a color value equivalent to 7.7 mm. (CR–MO)
0.40 " " " " " " " 15.4 " (CR–MO)

The color was exactly like that produced by histamine. This, by table, is equivalent to 0.005125 gm. of histamine dichloride for the entire test liquid. From this, the amount of histamine dichloride previously found to be present in this sample of Witte's peptone—0.0001675 gm.—must be subtracted, which leaves 0.00496 gm. as the amount of the *introduced* histamine dichloride that was recovered, which is 99.3 per cent.

FIG. 4. Contraction of excised virgin guinea pig uterus produced by the silver precipitate from 1 gm. of Witte's peptone. The presence of histamine was also indicated by the colorimetric determination.

CONCLUSIONS.

1. A typical peptone shock is obtained by the injection of a histamine-free peptone; hence peptone shock and histamine shock are not identical.

2. A histamine-free peptone contracts the excised virgin guinea pig uterus.

3. Pure peptone contains a substance or substances, apparently basic in character, that are not destroyed by boiling with hydrochloric acid, that are precipitated by phosphotungstic acid, extracted by amyl alcohol from an alkaline aqueous solution, that combine with sulfuric acid to give salts that are soluble in water, and that are capable of producing a fall in blood pressure. This fall in blood pressure is, however, far inferior to the similar fall in blood pressure obtained with the equivalent quantity of peptone. In the above respects the substances are similar to histamine. There are, however, two very marked differences between the above substances and histamine; they give no color with *p*-phenyldiazonium sulfonate and they do not contract the excised virgin guinea pig uterus.

4. A sample of Witte's peptone—100 gm.—was found to contain the equivalent of 0.00335 gm. of histamine dichloride.

STUDIES ON PROTEINOGENOUS AMINES.

XI. RESPONSE OF THE EXCISED UTERUS TO POTASSIUM, RUBIDIUM, AND CESIUM IONS.

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While we were searching for histamine in feces, blood, liver, and hypophysis, we noticed that the silver filtrate fractions,[1] although they could not have contained more than traces of histamine, frequently evoked a pronounced response from the excised virgin guinea pig uterus. Since this fraction always contains a small quantity of the nitrate ion, which is originally introduced as silver nitrate, we thought, at first, that this ion might be responsible for the physiological activity. To test the accuracy of this surmise, 1 cc. each of chemically pure 10 per cent solutions of sodium and potassium nitrate were separately introduced into the 100 cc. of Locke-Ringer solution in which the uterus was suspended. Fig. 1 illustrates the response obtained with the potassium nitrate solution. *A response was not obtained with the sodium nitrate solution.* From these results we concluded that the sodium and the nitrate ions did not stimulate the uterus muscle and that the response obtained with potassium nitrate was due to the potassium ion. This led naturally to an investigation of the effect of some of the other closely related metallic ions on the excised uterus.

Solutions of chemically pure sodium chloride, sodium nitrate, lithium chloride, ammonium chloride, potassium chloride, potassium nitrate, rubidium chloride, cesium chloride, calcium chloride, and magnesium sulfate were employed in these tests. *A response was obtained only with the potassium, rubidium, and*

[1] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1920, xliii, 547.

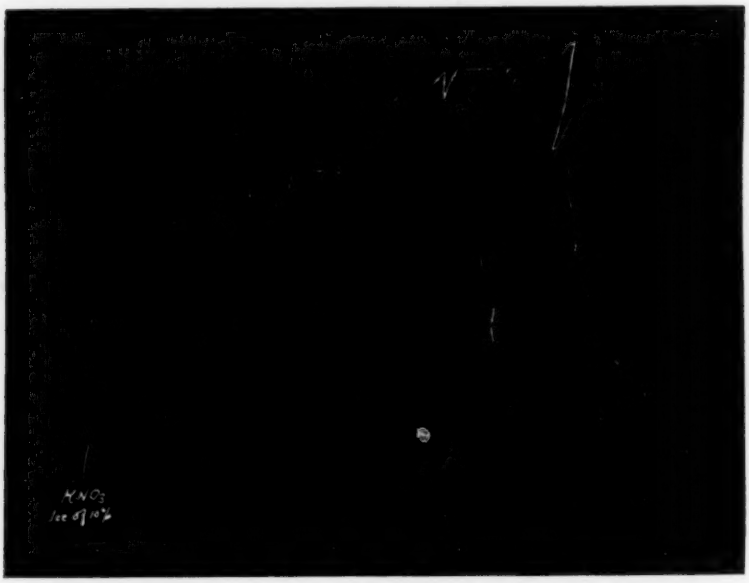

FIG. 1. Response of the excised virgin guinea pig uterus to potassium nitrate. The uterus was suspended in 100 cc. of a Locke-Ringer solution.

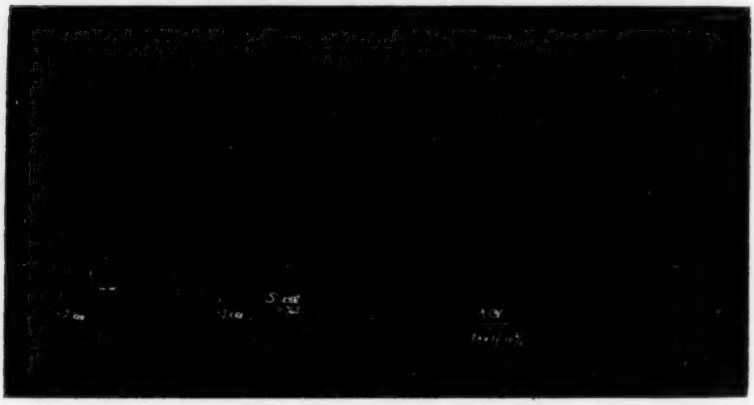

FIG. 2. Response of the excised virgin guinea pig uterus to various concentrations of potassium chloride. The uterus was suspended in 100 cc. of a Locke-Ringer solution.

FIG. 3. The response of the excised virgin guinea pig uterus to various concentrations of rubidium chloride. The uterus was suspended in 100 cc. of a Locke-Ringer solution.

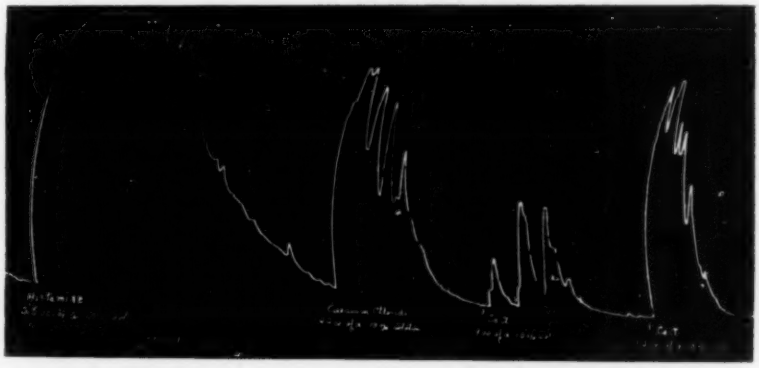

FIG. 4. Response of the excised virgin guinea pig uterus to various concentrations of cesium chloride. The uterus was suspended in 100 cc. of a Locke-Ringer solution. The first curve, a typical histamine tracing, is introduced for the sake of comparison.

cesium salts. Fig. 2 shows the response obtained when 0.20, 0.30, 0.50, and 1.0 cc. of a 10 per cent solution of potassium chloride were introduced into the 100 cc. of Locke-Ringer solution in which the uterus was suspended. Fig. 3 illustrates the response obtained when 0.05, 0.10, 0.40, 0.80, and 1.6 cc. of a 10 per cent solution of rubidium chloride were introduced into the 100 cc. of Locke-Ringer solution in which the uterus was suspended. The last two tracings in this figure illustrate the response evoked by equivalent quantities of potassium and rubidium salts. Fig. 4 shows the response obtained when 1.0, 1.5, and 2.2 cc. of a 10 per cent solution of cesium chloride were introduced into the 100 cc. of Locke-Ringer solution. The first tracing in this figure was obtained by introducing 0.50 cc. of a 0.01 per cent solution of histamine dichloride into the 100 cc. of Locke-Ringer solution and it is inserted here merely for comparison.

Since no response was obtained with equivalent quantities of sodium chloride, sodium nitrate, ammonium chloride, lithium chloride, calcium chloride, and magnesium sulfate it is safe to say that the Cl, NO_3, SO_4, NH_4, Li, Ca, and Mg ions have no stimulating action upon the uterus muscle and that the potassium, rubidium, and cesium ions are responsible for the response obtained.

CONCLUSIONS.

Potassium and rubidium salts in sufficient concentration—about N/75 in each case—produce a tonic contraction of the virgin guinea pig uterus from which the muscle does not recover until the stimulating ions are removed. The minimum effective concentration of the potassium ion is N/150 and of the rubidium ion is N/1,210 from which it appears that the rubidium is approximately eight times as active as potassium. The minimum effective concentration of cesium appears to be about N/150; but the response obtained with this ion is neither so vigorous nor so permanent as that obtained with either potassium or rubidium.

SOME NUTRITIVE PROPERTIES OF NUTS; THEIR PROTEINS AND CONTENT OF WATER-SOLUBLE VITAMINE.*

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(Received for publication, June 25, 1920.)

The extensive series of metabolism experiments on fruitarians and nutarians in California reported by Jaffa (1901, 1903) indicated that nuts used as a substantial component of the diet are quite thoroughly digested and are of a higher nutritive value than is popularly attributed to them. The same conclusion was reached by Cajori (1918) through digestion trials of raw and heated nuts, nut butters, and nut pastes. In his experiments the coefficient of digestibility for nut proteins and carbohydrates fell well within the range of the protein coefficient for a mixed diet. There was no indication that nuts are especially resistant to the digestive functions of the alimentary canal; so that the conclusion seemed justified that nuts are valuable foods and, if eaten properly and used in the diet with due regard to their concentrated make-up, are on a physiological par with common staple articles of the diet. A more detailed investigation of the nutritive properties of nuts has, however, hitherto been wanting.

The Nutritive Value of Nut Proteins.

It is clearly recognized that individual proteins may vary greatly in the proportions of the various amino-acids which may be obtained from them and that their dietary value depends in

* The data in this paper are taken from the dissertation presented by the writer for the degree of Doctor of Philosophy, Yale University, 1920. A part of the expense of the investigation was defrayed by a grant from the Russell H. Chittenden Research Fund for Physiological Chemistry.

large part on the character of this amino-acid yield. An appreciation of the significance of these nutritive differences is largely due to the extended studies of Osborne and Mendel and others, who have demonstrated in the case of the albino rat that the naturally occurring proteins differ widely in their efficiency for maintenance or growth.

With reference to *the chemical make-up of the proteins of nuts*, Osborne and Clapp (1907, 1907–08) have studied the products of acid hydrolysis of amandin and excelsin, the principal proteins of the almond and Brazil nut, respectively. Osborne and Harris (1903, *a*, *c*, *d*) compared the distribution of basic amino-acid nitrogen in the globulins of the almond, Brazil nut, black walnut, English walnut, butternut, and filbert, and have shown that the Hopkins-Cole test for tryptophane is strongly positive with all these proteins. From the investigations of these authors, the black walnut, English walnut, and butternut would seem to contain very similar, if not identical, globulins as their principal proteins. The name juglansin has been given to this globulin of the juglans species of nut. Osborne and Harris (1903, *b*) found the only essential difference between corylin, the globulin from the filbert or hazel nut, and juglansin to be a higher content of amide nitrogen in corylin and a difference in the specific rotation of the two proteins. Using Van Slyke's newer method for protein analysis, Nollau (1915) has analyzed the pecan nut, peanut, black walnut, and hickory; Johns and his collaborators (1917, 1919, *a*) applied the same method to a study of the globulins of the peanut and coconut. In all these studies, the relatively high content of basic amino-acids found and the presence of tryptophane suggest that these nuts are likely to be sources of complete protein. Speaking of the peanut, Johns says: "the relative high percentage of lysine in the proteins of the peanut indicates that this seed might be used to advantage in supplementing diets deficient in lysine."

This conclusion, based on chemical analysis, that nut proteins are of a high biological value, has been verified by the feeding experiments of Johns and coworkers (1919, *b*) with the coconut, and Daniels and Loughlin (1918) with the peanut. These investigators observed normal growth in young rats on diets in which the coconut and peanut furnished the sole source of protein. Osborne and Mendel (1912) have maintained rats over long periods in which the protein of the dietary was derived from excelsin of the Brazil nut.

Feeding Experiments with Rats.

The experience of numerous investigators in experiments involving growth and maintenance has demonstrated the many advantages in the use of the rat as an experimental animal in nutrition studies.

Following the general technique of Osborne and Mendel, as recently described by Ferry (1920), we have extended the feeding observations on nuts to include the almond, English walnut, filbert, pecan, and pine nut. Young rats were fed on diets complete in respect to every known dietary essential for growth, provided the proteins were of a character to support growth. Nut proteins made up approximately 18 per cent of the diets. It is well recognized that rats will grow to adult size at a normal rate on this level of protein intake, if the protein is "complete" from a nutritive point of view; *i.e.*, furnishes all the essential amino-acids in suitable amounts.

There are not many nuts that contain over 3 per cent of nitrogen. Therefore in order to prepare a diet containing 18 per cent of protein (*e.g.* 2.9 per cent N $\times$ 6.25) and still insure, in addition to the protein, the presence of adequate quantities of the recognized dietary essentials, fat-soluble and water-soluble vitamines and inorganic salts, concentration of the protein of certain of the nuts was necessary. To effect this, the shelled nuts were passed through a meat grinder and then subjected to pressure in a tincture press. This process removed considerable quantities of oil from the nut, and nitrogen determinations of the residual press cake indicated that the protein content had been sufficiently increased to incorporate in the diet at the desired 18 per cent protein level.

Charts 1 and 2 show the growth curves of albino rats on diets in which the essential source of protein was derived from nuts. At the time these experiments were inaugurated the presence of water-soluble vitamine in the nuts used had not been demonstrated. Dried brewer's yeast was added to the diets, to the extent of 2 per cent, to make sure that they contained adequate quantities of this dietary essential. The possibility of the supplementary action of the yeast in these diets must be recognized. Osborne and Mendel (1919, *a*) found yeast to be adequate for growth when fed as the sole source of protein. However, the amount of protein derived from yeast fed in a concentration of 2 per cent and its supplementing action cannot be large. In Osborne and Mendel's successful experiments the dried yeast furnished 30 and 40 per cent of the total food intake. Neuberg (1916) estimates that 52 per cent of yeast nitrogen is non-protein

TABLE I.

Weekly Food Consumption on Nut Diets.

Week.	Almond.		English walnut.	Filbert.		Pecan.		Pine nut.		Casein.	
	Average for Rats 10, 11, 12.		Average for Rats 13, 14, 15.	Average for Rats 16, 17.		Rat 30.	Rat 31	Rat 32.	Rat 33.	Rat 7.	Rat 8.
	gm.		gm.	gm.		gm.	gm.	gm.	gm.	gm.	gm
1	45		50	40		43	40	39	42	41	41
2	41		40	40		60	40	45	42	44	42
3	33		42	39		48	35	39	38	53	48
4	42		47	44		46	34	35	44	50	52
5	44		51	44		47	41	53	50	54	54
6	50		45	41		47	33	44	37	61	59
7	55		50	54		44	40	48	43	59	57
8	49		50	41		43	41	44	48	61	63
9	51		48	58		44	40	53	57	63	54
10	53		48	45		55	59	57	52	54	47
11	51		48	54		52	50	59	46	61	58
12	51		46	56		52	51	53	55		
13	50		47	45		54	47	45	50		
14	52		42	38				46	52		
15	56		43	53				45	45		
16	66		43	53				48	55		
17	57		41	50				54	50		
18	48		38	41				63	62		
	Rat 10.	Rat 12 + young.		Rat 16 + young.	Rat 17.						
19	65	68		37	41			65	66		
20	50	76		69	31			65	55		
21	49	60		69	41			60	50		
22	49	49		60	40			68	59		
23	45	49		65	41			65	53		
24	61	61		85	42			63	54		
25	54	62		98				77	45		
26		84		91				80			
27		104		46*				99			
28		122		42							
29		117									
30		45*									
31		44									

* Young removed.

nitrogen. If this estimation is correct, yeast proteins made up but 4 per cent of the total protein of our diets.

Table I gives the weekly food consumption of the rats on the nut diets. The average normal intakes of animals *of the same weight* on a comparable casein diet are on record (Osborne and Mendel, 1915). The animals, as a rule, ate less of the nut food than did Osborne and Mendel's rats on the casein diet similar in composition to that from which their normal intake was calculated. The nut foods, consisting of a large quantity of a fat-rich press cake probably were of a higher calorific value than the casein diet. This fact would explain these differences, as the quantity of food eaten by rats which limit their intake to their energy requirement is largely determined by the calorific value of the food.

Examination of the growth curves shows that growth resulted at a normal rate on all diets except the one in which the pecan supplied the protein. The animals on this diet grew at a rate about two-thirds normal during the 9 weeks that they were on this diet. When casein was introduced into the pecan diet in such a way that one-third of the pecan protein was replaced by casein, a marked rise in the rate of growth was noted. This increased growth may have been due either to a supplementing action of the casein or to an increased food intake resulting from some favorable modification in the taste or character of the pecan diet on addition of casein.

More experiments are necessary before final conclusions can be drawn regarding the comparative efficiency of the proteins of the pecan.

Growth of the second generation of young rats on the nut diets is shown in Chart 3.

With the limited number of observations that we have recorded it is not possible to make numerical comparisons of different nut proteins, nor were our experiments planned to demonstrate the maximum growth-promoting power of nut proteins. But, accepting the opinion of Osborne and Mendel (1920, *a*), ". . . . that if an animal is able to attain adult size upon a diet which furnishes protein from a single source, the nutritive value of this protein is clearly established," we conclude from our experiments that the almond, English walnut, filbert, and pine nut are sources of protein adequate for nutrition.

Nuts as Sources of Water-Soluble Vitamine.

Numerous feeding experiments, carried out during the last few years, have demonstrated that vitamines are widely distributed among plant products. The cereals, vegetables, and forage crops have been investigated in some detail as to their content of the water-soluble and fat-soluble vitamine. Nuts, as a class, however, have received little attention with respect to the presence in them of these important dietary essentials.

Halliburton and Drummond (1917), studying various naturally occurring fats, failed to obtain normal growth in rats when the butter of their diets was replaced by coconut oil, peanut oil, or walnut butter. Hence the fat-soluble vitamine would seem to be absent in nut oils. Daniels and Loughlin (1918) showed that peanuts are lacking in the fat-soluble factor but that considerable amounts of the water-soluble vitamine are present; and Johns, Finks, and Paul (1919) observed satisfactory growth in young rats on diets in which coconut meal was supplemented by butter and inorganic salts only and therefore represented the sole source of water-soluble vitamine. Mackenzie-Wallis (1918) has reported that a flour made from ground peanuts was antiscorbutic. Grieg (1918) used ground nut meal biscuits with some success as a therapeutic agent in experimental beri-beri.

Feeding Experiments with Rats.

We have studied many of the more common nuts, that have not previously received attention, as possible sources of water-soluble vitamine and have planned our experiments with a view to being able to obtain an approximate quantitative idea of the comparative distribution of this essential food factor among members of this class of plant products.

Two methods are available for such trials. An animal may be placed on a diet deficient in respect to the water-soluble factor. On such a diet, the animal, if it is still small, will cease to grow and in any event will, in a short time, begin to decline rapidly in weight, a condition that terminates in death unless a change is made in the diet. If a product containing sufficient quantities of water-soluble vitamine is added to the diet before the animal is permanently injured by undernourishment, a rapid recovery of weight and nutritive well-being take place. The second method depends on the fact that normal growth can be observed in young rats only when the product to be tested is available in sufficient

quantities to supplement an otherwise complete diet with an abundance of water-soluble vitamine.

In the experiments reported below, both methods were employed. The basal diet used, consisting of casein, butter fat, inorganic salts, starch, and lard, was deficient in water-soluble vitamine as evidenced by the rapid decline of animals when placed on this food mixture exclusively. Control experiments indicated that the casein diet was complete with respect to all other food essentials, as normal growth was observed when small amounts of brewer's yeast, known to be rich in water-soluble vitamine, were added to this diet.

In the majority of our trials weighed quantities of the nut to be tested were fed daily apart from and in addition to the basal diet. This method, introduced by Osborne and Mendel (1919, *b*), insures a constant intake of the product under investigation and enables a comparison to be made of the potency of the foods consumed in like amounts. By varying the daily dosage, further evidence in respect to the comparative quantities of the water-soluble vitamine present can be obtained.

The nuts were shelled and care was taken to remove all fragments of the shell and the coarser parts of the integument. In the case of the almond, the nut was blanched. The chestnuts used in the later trials (Charts 6 and 8) were fed after completely freeing them from the adhering integument. We found that the rats, at least after the first few days, readily ate the nuts and, as a rule, consumed the entire nut allowance as soon as it was placed in the cage. The only failure to eat that we experienced was in the case of the Brazil nut. It was found necessary to incorporate this nut in the basal diet before the animals would consume it in the desired amounts.

The outcome of the restorative trials is shown in Charts 4, 5, and 6 and Table II. A daily dosage of 2 gm. of hickory nut, pine nut, and pecan, respectively, or the inclusion of 27 per cent of almond, or filbert, and 19 per cent of English walnut in the food sufficed to enable animals that had declined on the basal diet to recover rapidly their nutritional well-being and resume growth. 1 gm. of English walnut, black walnut, hickory nut, or pine nut proved to be an equally efficient restorative agent. Partial recovery and slow growth resulted from daily consump-

tion of 1 gm. of almond and 0.5 gm. of chestnut, English walnut, or pecan in addition to the basal diet. These charts show that all the nuts tested are comparatively rich in water-soluble vitamine. The chestnut, English walnut, and pecan appear to be especially potent, daily doses of 0.5 gm. sufficing to permit slow growth. The animals receiving 1 gm. daily of either

TABLE II.

Weekly Consumption of Basal Diet and Nuts.

Week.	Hickory nut.				Pine nut.				Almond.			
	Rat 4.	Rat 6.	Rat 38.	Rat 39.	Rat 8.	Rat 9.	Rat 34.	Rat 35.	Rat 2.	Rat 18.	Rat 19.	Rat 20.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	53	80	43	43	58	49	22	46	71	43	47	48
2	52	68	32	37	55	45	13	37	63	41	41	36
3	42	65	49	29	56	44	31	53	55	43	39	39
4	59	58	61	36	45	37	74	78	32	45	48	46
5	49	61	62	58	31	29	78	60	65	25	38	29
6	80	101	63	55	46	32	73	55	72	27	27	22
7	62	82			79	58			69	44	42	41
8	62	78			59	58			71	43	49	36
9	58	73			60	63			59	43	51	40
10	68	78			60	58				30	40	40
11										56	42	51
12										54	56	56
13												

	English walnut.					Pecan nut.			
	Rat 3.	Rat 7.	Rat 24.	Rat 30.	Rat 31.	Rat 21.	Rat 22.	Rat 25.	Rat 26.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	74	61	47	52	45	51	38	54	39
2	59	53	52	57	53	49	41	70	50
3	46	49	42	52	37	42	32	64	50
4	32	42	38	36	22	34	24	53	44
5	88	30	35	35	28	61	46	32	29
6	71	30	67	53	30	59	42	23	24
7	55	23	68	57	31	45	31	47	40
8	55	60	61	58	48	58	37	50	49
9	67	57		53	57	54	35	54	42
10	77	73		59	63	60	35	44	44
11		59		73	51			52	62
12		53		53	48			59	69
13								60	60

TABLE II—*Concluded.*

Week.	Chestnut.			Black walnut.		Filbert.
	Rat 27.	Rat 28.	Rat 29.	Rat 36.	Rat 37.	Rat 1.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	66	55	62	50	47	84
2	55	56	63	39	34	57
3	32	50	32	31	44	45
4	21	30	27	45	19	36
5	19	19	26	48	35	112
6	43	43	50	52	45	106
7	51	38	47	63	44	80
8	43	34	43			82
9	41	37	44			
10	45	39	45			
11	55	45	56			
12	55	50	59			
13	58	65	67			

English walnut or pine nut showed as rapid recovery as in any trial where the daily dose of other nuts was 2 gm. A comparison of the growth curves indicates that the almond was not so efficient as a source of water-soluble vitamine. 1 gm. of this nut was less effective as a restorative agent than 0.5 gm. of the pecan or chestnut.

Osborne and Mendel (1920, *b*) believe that animals which have suffered a decline due to a deficiency of water-soluble vitamine may become so badly nourished that failure to effect prompt resumption of growth may sometimes be ascribed to the condition attained by the animal rather than to a lack of the vitamine in the tested product used as a supplement to the deficient diet. The rats used in our experiments were subjected to quite similar degrees of undernutrition and it would seem that the variations in rapidity of recovery can be interpreted, in our experiments, as being due to differences in the quantity of water-soluble vitamine present in different nuts rather than to a radical difference in the nutritive condition of the animals at the time that the nut feeding was inaugurated. The weekly food intakes are shown in Table II.

The growth curves of animals on diets in which nuts, supplied in different daily doses, furnished the sole source of water-soluble

vitamine are shown in Charts 7 and 8. Here again, the richness of nuts in this vitamine is demonstrated. Normal growth was observed when the basal diet was supplemented daily by 2 gm.

TABLE III.

Weekly Consumption of Basal Diet and Nuts.

Week.	Almond.			English walnut.			Brazil nut.			Black walnut.	
	Rat 18.	Rat 19.	Rat 20.	Rat 21.	Rat 22.	Rat 23.	Rat 24.	Rat 25.	Rat 26.	Rat 34.	Rat 35.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	44	43	43	48	47	41	48	50	43	77	55
2	42	43	42	43	42	39	46	47	48	68	67
3	47	43	43	43	39	38	48	31	34	71	55
4	46	43	47	47	44	37	46	45	33	70	60
5	40	41	47	51	38	40	43	38	43	54	55
6	39	37	41	51	35	40	43	38	35	46	64
7	44			61	42	43		58		37	54
8	42			45	35	43		52		40	50
9				51	36	47		54		34	43
10										31	62
11										30	64

	Chestnut.					Pecan.		Yeast.
	Rat 27.	Rat 28.	Rat 29.	Rat 38.	Rat 39.	Rat 36.	Rat 37.	Rat 9.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	53	47	50	60	70	66	73	41
2	51	51	50	57	57	56	55	41
3	51	44	51	54	57	55	54	45
4	52	45	53	47	63	48	66	47
5	51	52	68	52	61	52	62	43
6	42	45	45	60	61	54	55	39
7	60	53	57	55	59	55	57	49
8	56	48	55	55	54	51	59	50
9	66	55	62	47	51	57	46	56
10				51	48	58	47	
11				48	55	49	55	

of the almond, English walnut, chestnut, Brazil nut, or black walnut. When the daily ration of the nut was 1 gm., the pecan and chestnut furnished sufficient quantities of this dietary factor for normal growth.

The weekly food intakes are shown in Table III. The characteristic deline in food consumption during the period when there was insufficient water-soluble vitamine in the food, and the prompt recovery of appetite and resumption of eating when the vitamine was added to the diet, are to be noted.

Nut Proteins and Milk Production.

The present experiments with nut diets have furnished an opportunity to test some of the relations of the dietary proteins to milk production, inasmuch as a number of the female rats under observation were bred in the course of the feeding trials. If the mammary gland has at best a limited power, if any, of synthesizing the essential amino-acid precursors of its protein complexes (McCollum and Simmonds, 1918), the importance of furnishing a proper source of amino-acid groups in the ration during lactation is evident.

In studying the lactation of dairy cows, Hart and Humphrey (1915, 1916, 1917, 1918), among others have found wide differences in the efficiencies of various protein mixtures as producers of milk. Not only the quantity but also the biological quality of the proteins in the ration affect the character of the mammary secretion. Likewise Hoobler (1917, *a*, *b*) has concluded from studies of wet nurses that, as a rule, animal proteins are more efficient than vegetable proteins for the elaboration of human milk. However, nut proteins were an exception to this generalization in that diets containing almonds, English walnuts, pecans, and peanut butter as a source of protein proved to be as suitable for milk production as diets which furnished protein from animal sources. In other words, nuts seemed to furnish the nitrogenous complexes necessary for the elaboration of milk as effectively as any other type of protein.

Judging the character of milk production by the ability of the mother to nurse her young successfully, we have observed satisfactory mammary function in rats, on diets containing the almond, English walnut, pine nut, and filbert as the essential source of protein in the ration. Some loss of weight of the mother rat occurred during the nursing period in the experiments with the almond, English walnut, and filbert, but tissue disintegration during the temporary decline could scarcely have furnished enough indispensable nutrient units to account for the newly synthesized milk protein sufficient in quantity to have produced the increments in weight observed in all the young.

SUMMARY AND CONCLUSIONS.

Satisfactory growth was observed in young rats on diets in which the almond, English walnut, filbert, and pine nut, respectively, furnished the essential source of protein in the ration.

Normal growth can be secured when rats are fed upon otherwise adequate diets containing the almond, English walnut, black walnut, Brazil nut, chestnut, or pecan as the sole source of water-soluble vitamine. Animals which have declined on a diet devoid of water-soluble vitamine promptly recover when the almond, English walnut, filbert, hickory, pine nut, chestnut, or pecan is introduced in the diet. These observations indicate that nuts are sources of abundant quantities of water-soluble vitamine.

The proteins of the almond, English walnut, pine nut or filbert furnish the necessary nitrogenous complexes for the elaboration of milk in rats.

I desire to express my hearty thanks to Professor Lafayette B. Mendel, who suggested this study to me, for his helpful advice and criticism during the course of the investigation.

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CHART 1. Growth of young rats on diets in which the English walnut, pine nut, and pecan, incorporated in the diet in the form of a press cake, furnished the essential source of protein. A casein diet of similar composition, except for protein, served as a control experiment. The broken line in the curves of Rats 30 and 31 indicate the period when the pecan diet was replaced by a mixture of one part casein diet and two parts pecan diet.

The composition of the casein and nut diets was as follows:

Casein Diet.

Rat 7.	*per cent*
Casein	18
Salt mixture*	4.5
Starch	50.5
Butter fat	9
Lard	18
Brewer's yeast, dried	200 mg. daily.

* The salt mixture used in all the diets of the feeding trials is that described by Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 317.

English Walnut Diet.

Rats 13, 14, 15.	*per cent*
English walnut press cake, 3.5 per cent N	82
Salt mixture	3
Starch	4
Butter fat	5
Lard	4
Brewer's yeast, dried	2

Pine Nut Diet.

Rats 32, 33.	*per cent*
Pine nut	50
Salt mixture	3
Starch	36
Butter fat	5
Lard	6
Brewer's yeast, dried	200 mg. daily.

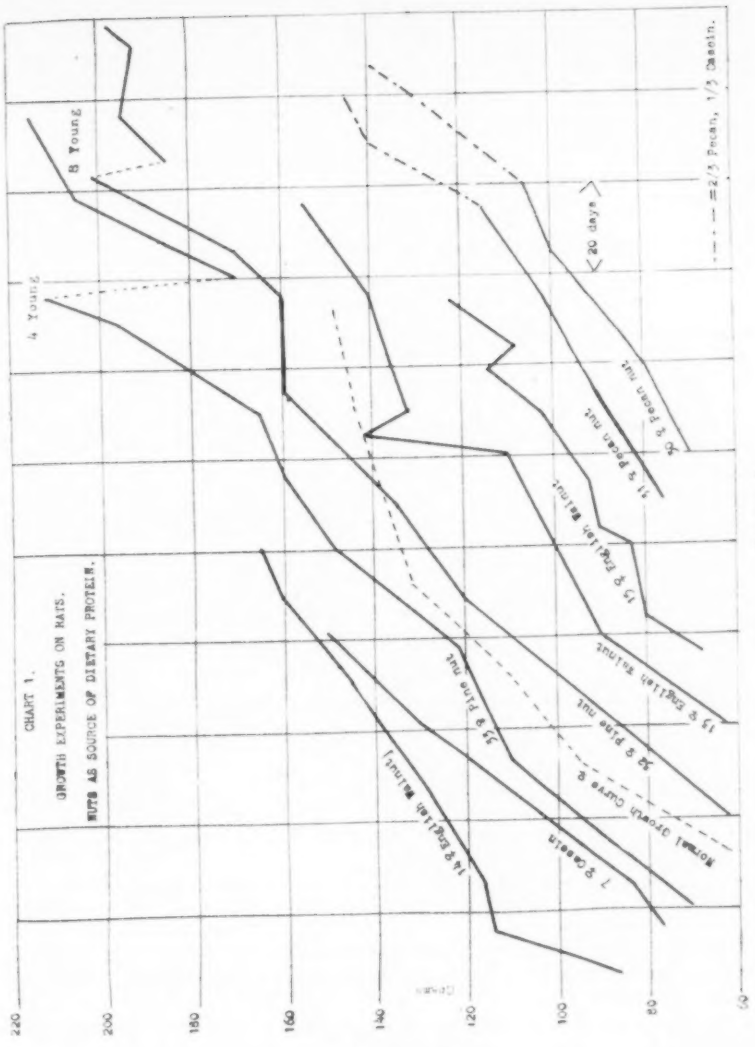

CHART 1.

Pecan Diet.

Rats 30, 31.

	per cent
Pecan press cake, 3.6 per cent N	80
Salt mixture	3
Starch	7
Butter fat	5
Lard	5
Brewer's yeast, dried	200 mg. daily.

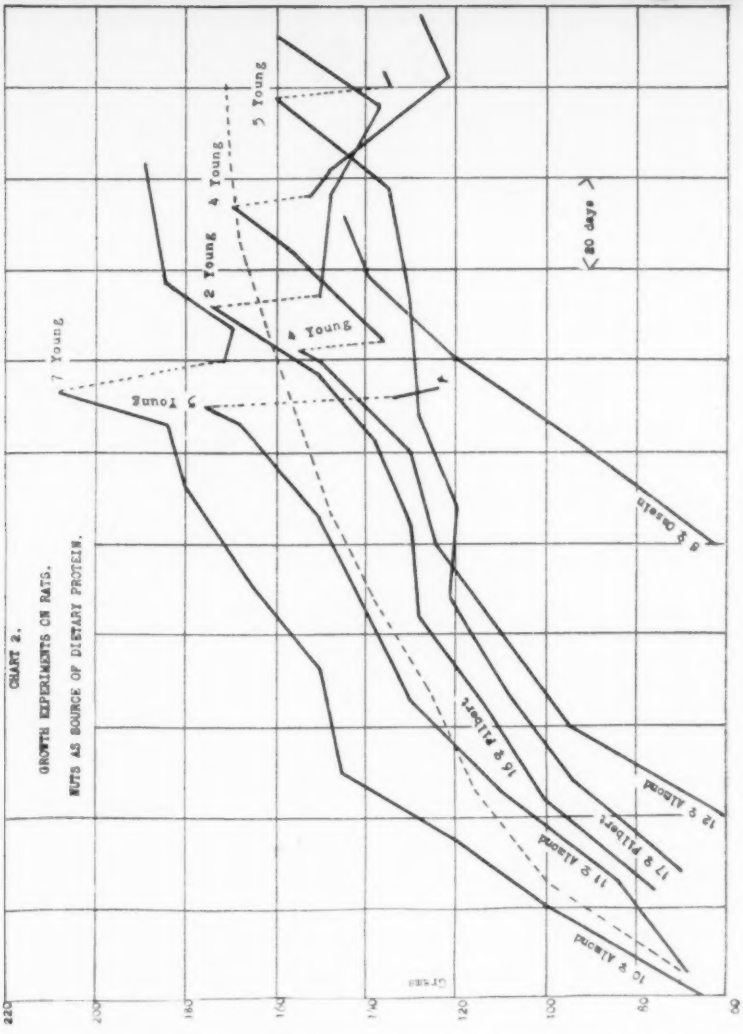

CHART 2.

CHART 2. Growth of young rats on diets where the almond and filbert, incorporated in the diet in the form of a press cake, furnished the essential source of protein.

The composition of the casein diet was the same as given in the description of Chart 1. The composition of the nut diets was as follows:

Almond Diet.

Rats 10, 11, 12.	*per cent*
Almond press cake, 5.0 per cent N	58
Salt mixture	3
Starch	12
Butter fat	5
Lard	20
Brewer's yeast, dried	2

Filbert Diet.

Rats 16, 17.	*per cent*
Filbert press cake, 3.6 per cent N	80
Salt mixture	3
Starch	3
Butter fat	5
Lard	7
Brewer's yeast, dried	2

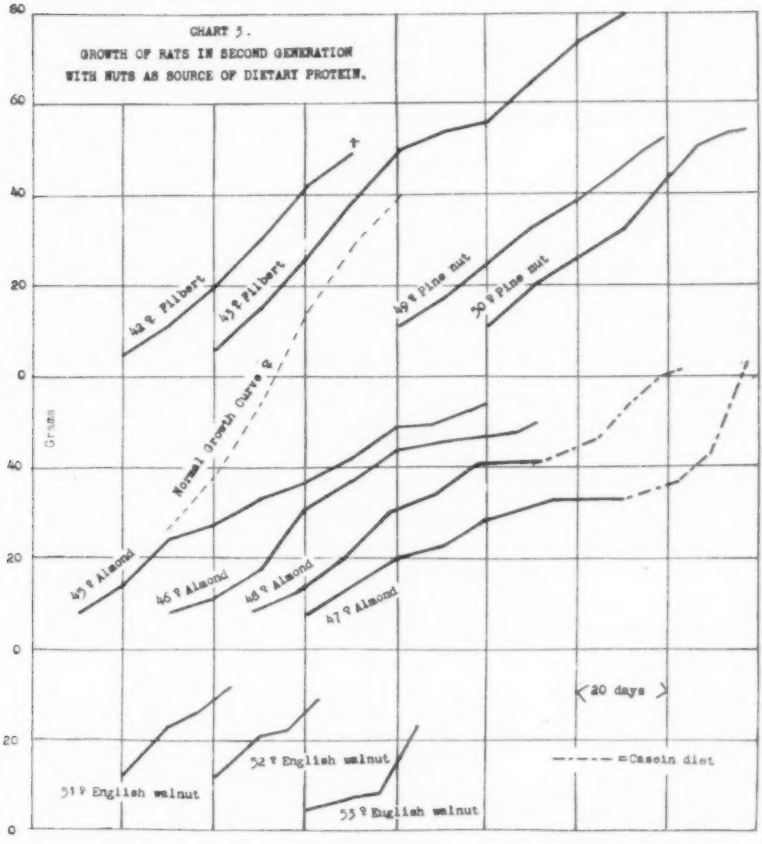

CHART 3. Growth of the second generation of young rats on diets in which almond, English walnut, filbert, and pine nut, incorporated in the diet in the form of a press cake, furnished the essential source of protein. The broken line in the curves of Rats 47 and 48 indicates the period when the almond diet was replaced by the casein diet and 100 mg. of dried yeast daily.

The composition of the casein and nut diets was the same as described in Charts 1 and 2.

CHART 4. Recovery of growth of animals which had declined on a diet devoid of water-soluble vitamine, on the addition of the almond, filbert, hickory nut, pine nut, pecan, and English walnut as supplements to the basal diet.

The animals receiving the hickory nut, pine nut, and pecan were given 2 gm. of the nut daily.

The almond, filbert, and English walnut, respectively, were incorporated in the diet, one part of nut diet and two parts of basal being thoroughly mixed together. In such a mixture the almond and filbert nut furnished 27 per cent of the diet and the English walnut 19 per cent.

The broken line on the curves denotes the period when the animals were on the basal diet.

The composition of the diets was as follows:

Basal Diet.

	per cent
Casein	18
Salt mixture	4.5
Starch	50.5
Butter fat	9
Lard	18

Almond Nut Diet.

Rat 2.	per cent
Almond press cake	58
Salt mixture	3
Starch	14
Butter fat	5
Lard	20

Filbert Diet.

Rat 1.	per cent
Filbert press cake	80
Salt mixture	3
Starch	5
Butter fat	5
Lard	7

English Walnut Diet.

Rat 3.	per cent
English walnut press cake	82
Salt mixture	3
Starch	6
Butter fat	5
Lard	4

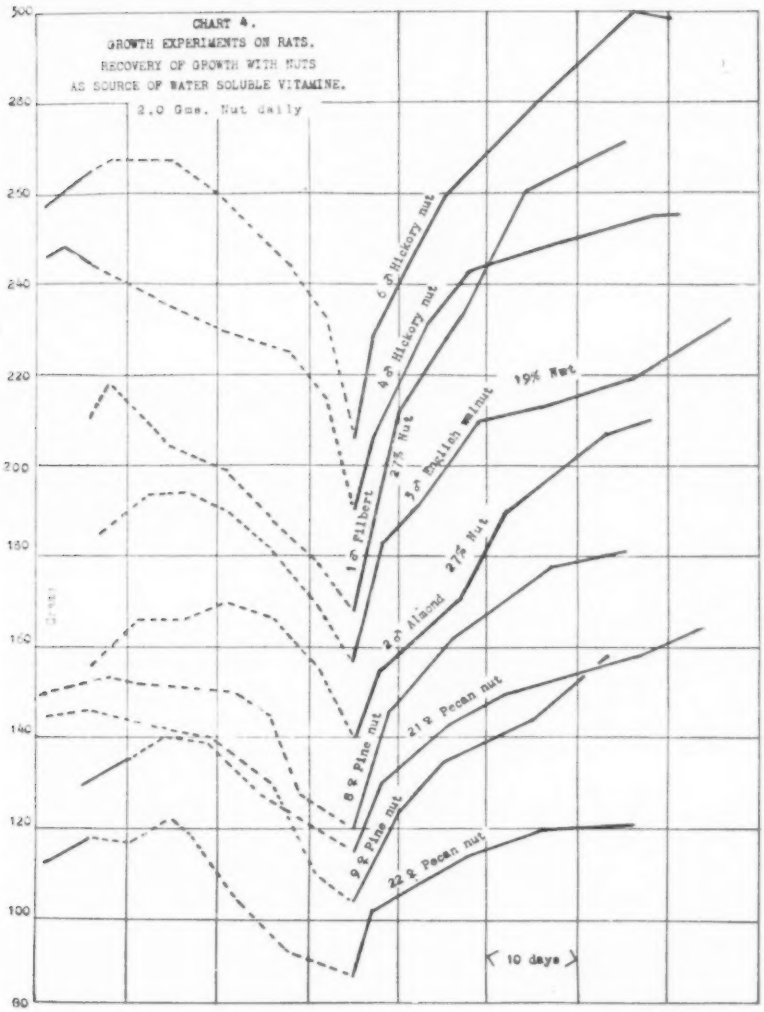
CHART 4.
GROWTH EXPERIMENTS ON RATS.
RECOVERY OF GROWTH WITH NUTS
AS SOURCE OF WATER SOLUBLE VITAMINE.
2.0 Gms. Nut daily
300
280
260
240
220
200
180
160
140
120
100
80
6 ♂ Hickory nut
4 ♂ Hickory nut
1 ♂ Filbert
2.7% Nut
3 ♂ English walnut
19% Nut
2 ♂ Almond
27% Nut
8 ♀ Pine nut
21 ♀ Pecan nut
9 ♀ Pine nut
22 ♀ Pecan nut
10 days

CHART 4.

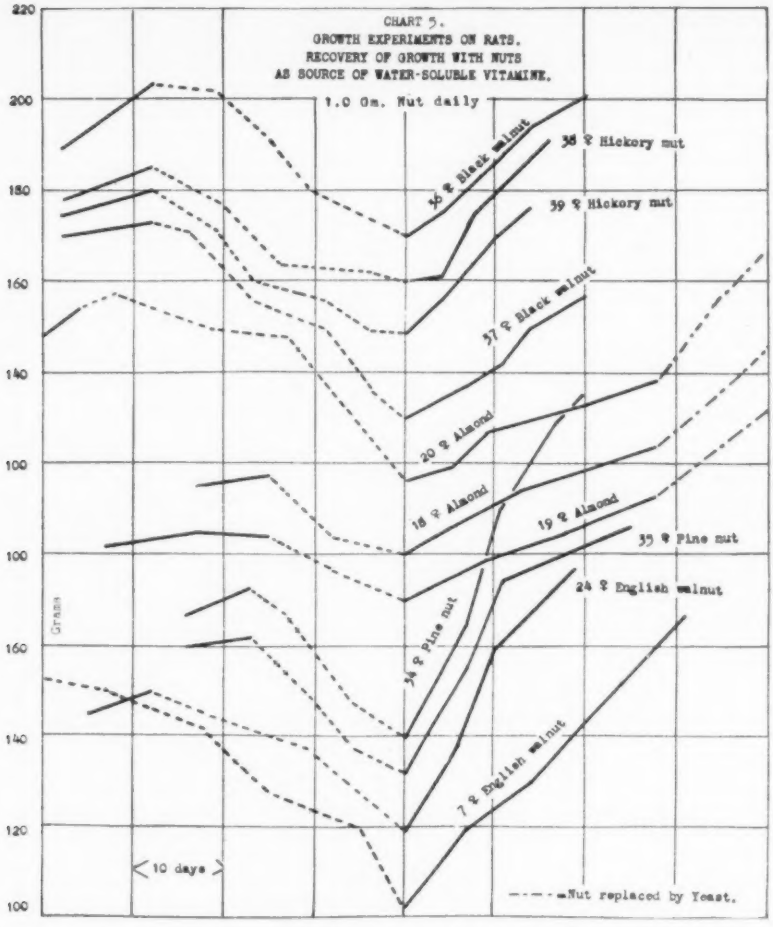

CHART 5. Recovery of growth of animals which had declined on a diet devoid of water-soluble vitamine, on the addition of almond, hickory nut, black walnut, pine nut, and English walnut as supplements to the basal diet.

The animals were given 1 gm. of nut daily.

The broken line of the curves denotes the period that the animals were on the basal diet.

The composition of the basal diet was the same as described in Chart 4.

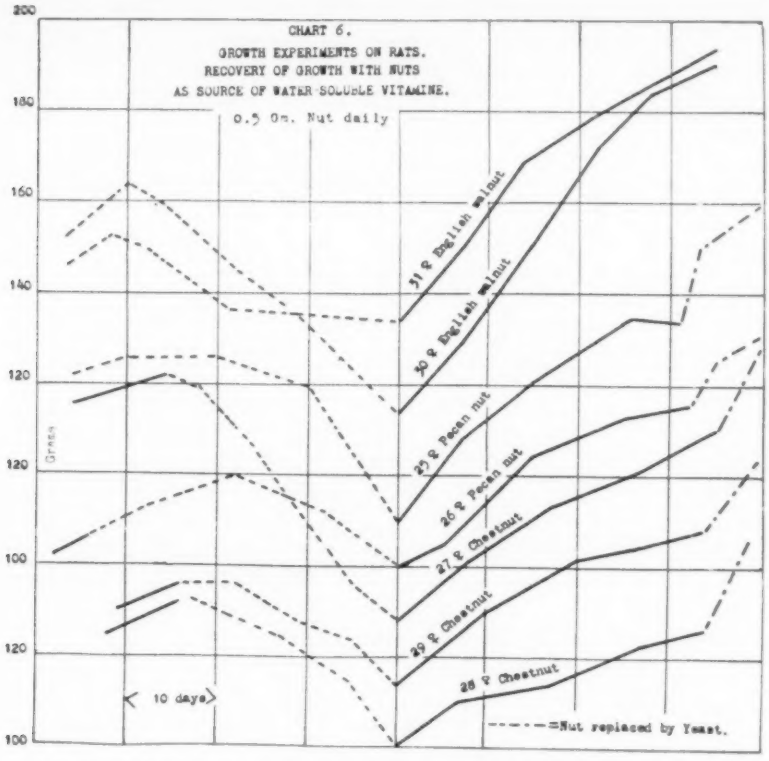

CHART 6. Recovery of growth of animals which had declined on a diet devoid of water-soluble vitamine, on the addition of the English walnut, chestnut, and pecan as supplements to the basal diet.

The animals were given 0.5 gm. of nut daily.

The chestnut was blanched before feeding.

The broken line indicates the period that the animals were on the basal diet alone.

The composition of the basal diet was the same as described in Chart 4.

CHART 7. Growth of young rats on diets where the almond, English walnut, chestnut, and Brazil nut furnish the sole source of water-soluble vitamine. 2 gm. of the nut were fed daily in addition to the basal diet. The basal diet was found to be inadequate to support growth unless supplemented by a source of water-soluble vitamine (see Charts 4, 5, 6).

The almond was blanched before feeding. The coarser parts of the integument of the other nuts were removed.

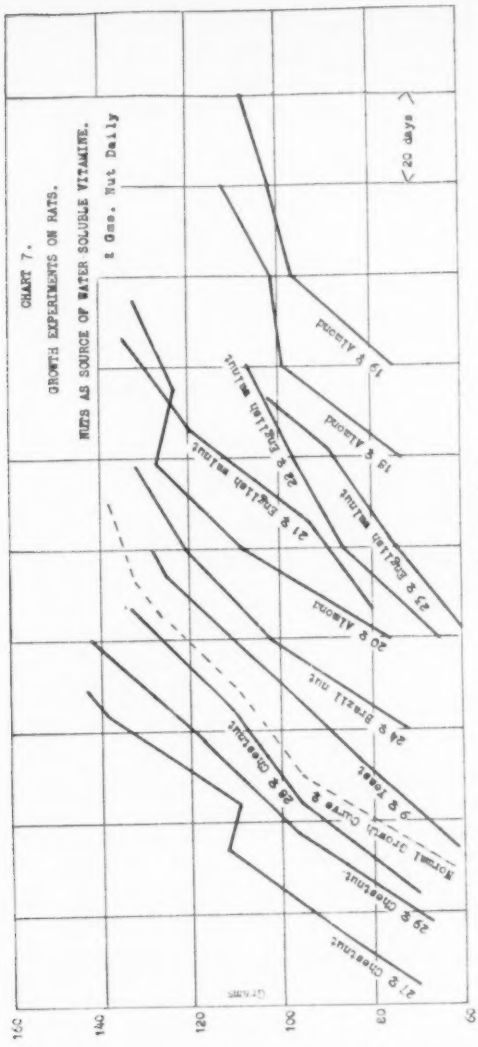

CHART 7.

The composition of the basal diet was as follows:

Basal Diet.

	per cent
Casein	18
Salt mixture	4.5
Starch	50.5
Butter fat	9
Lard	18

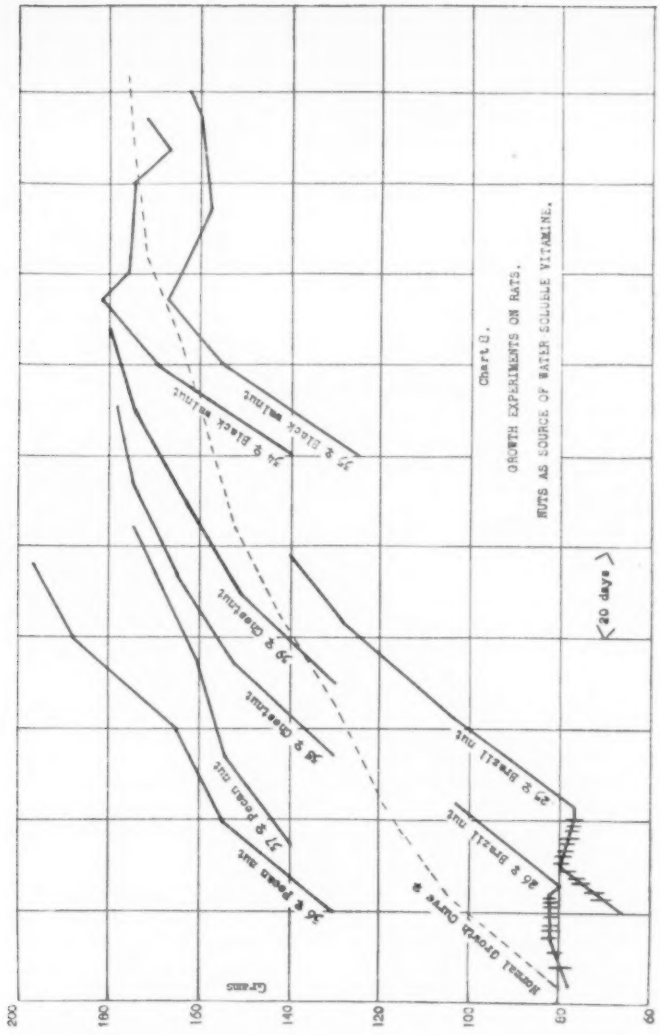

CHART 8.

CHART 8. Growth of young rats on a diet in which the black walnut, Brazil nut, pecan, and chestnut furnish the sole source of water-soluble vitamine. 2 gm. of the black walnut and Brazil nut were fed daily in addition to the basal ration. 1 gm. of the pecan and chestnut was fed daily in addition to the basal ration.

The chestnut was blanched before feeding. The coarser parts of the integument of the other nuts were removed.

The small vertical lines on the growth curves of Rats 25 and 26 indicate days when the animals failed to eat any of the nut given in addition to the basal ration. The prompt resumption of growth indicates the point where the diet was changed to a ration consisting of one part Brazil nut diet and two parts basal diet.

The composition of diets was as follows:

Basal Diet.

	per cent
Casein	18
Salt mixture	4.5
Starch	50.5
Butter fat	9
Lard	18

Brazil Nut Diet.

Rats 25, 26.	per cent
Brazil nut press cake	80
Salt mixture	3
Starch	7
Butter fat	5
Lard	5

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